UNIVERSIDADE DE LISBOA

Faculdade de Medicina Veterinária

REPRODUCTIVE MANAGEMENT IN CAPTIVE ELEPHANTS

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Abstract

Reproductive management in captive elephants

Elephants have been widely used by Humans for several centuries: for meat, as warriors through several kingdoms, for their heavy work power, for public entertainment, and for their unique tusks, leading them to be poached for the ivory trade. Nowadays we face the reality of a decreasing number of elephants in most of their range countries, leading them to be considered endangered (Asian) or vulnerable (African) to extinction.

Being charismatic mega-vertebrates, made them one of the most desired wildlife to keep and show in zoological collections.

Interdiction to the importation of wildlife was an important step, but with no more importation of individuals from the wild, the need to preserve the captive population became mandatory, and the zoological institutions make great efforts to maintain these animals in their collection and extend the conservation of these species. The inability to produce sufficient captive offspring and the continuous declining number in their natural habitat has urged research on elephant reproduction physiology.

Asian and African elephants reproduce well in the wild but due to historically poor reproductive performance under human care, most captive populations face the possibility of local extinction.

Besides logistical issues, elephant breeding in captivity faces management problems due to diseases, like ovarian and uterine pathologies and bull infertility.

Therefore, it is important to understand the anatomy, physiology and all associated pathologies which can lead to reproduction failure, and for the future management of captive elephant populations is fundamental to ensure that professional decisions are made.

Recent advances in endocrine monitoring and ultrasound imaging techniques allow researchers to understand the complex mechanisms that control reproduction in elephants, unique in several features.

In this thesis, I reviewed all relevant studies from 2000 to nowadays, with special emphasis to the African elephant. Reproductive breeding management considerations to the captive population of the Lisbon Zoo were derived. Finally, four clinical cases in elephant reproduction that were followed and assisted by the author are analysed and discussed.

Keywords: Reproductive management, Breeding, Elephants, Zoos, Conservation.

Resumo

Maneio reprodutivo de elefantes em cativeiro

Por muitos séculos, os elefantes têm sido utilizados pelo Homem: como produto de caça, soldados de guerra de diversos reinos, pela sua capacidade de trabalho pesado e pelas suas presas, levando a que sejam abatidos para o mercado de marfim. Atualmente, na maior parte da sua distribuição, o número de elefantes continua a decrescer o que levou à sua corrente classificação em "Vulnerável" (Africano) e em "Ameaça de Extinção" (Asiático).

Devido ao carisma destes mega vertebrados, os elefantes são um dos mais desejados animais para manter e exibir em Zoos. A interdição à importação de mais indivíduos do meio selvagem foi um passo importante para a conservação destas espécies, mas tornou a manutenção das populações cativas existentes uma prioridade e grandes esforços foram tomados pelas instituições de cativeiro. Um ponto fulcral tem sido o estudo da fisiologia reprodutiva dos elefantes, para que seja atingido um número de descendentes suficientes para manter estas populações. Tanto os elefantes asiáticos (*Elephas maximus*) como os elefantes africanos (*Loxodonta africana*) conseguem reproduzir-se com sucesso no meio selvagem mas, devido a uma história de baixa performance reprodutiva sob cuidados humanos, muitas das populações cativas correm o risco de extinção local. Para além de problemas logísticos, a reprodução de elefantes em cativeiro debate-se com questões de maneio devido a enfermidades, como patologias ováricas e uterinas e infertilidade no macho.

Compreender e tornar disponível as novas descobertas no ramo da anatomia, fisiologia e as mais comuns patologias associadas a falha reprodutiva tornou-se então uma prioridade para garantir que decisões ponderadas possam ser tomadas no maneio de elefantes cativos.

Avanços recentes em monitorização hormonal e em técnicas de ultrasom permitiu aos investigadores perceber os mecanismos complexos que controlam a reprodução nos elefantes, que apresentam variadas características únicas.

Posto isso, nesta tese, foram recolhidos e compilados todos os resultados relevantes publicados desde o ano 2000, com especial ênfase em dados relativos ao elefante africano.

Considerações sobre o maneio reprodutivo da população de elefantes Africanos existente no Jardim Zoológico de Lisboa são também abordadas. Por fim, quatro casos clínicos, auxiliados e seguidos pelo autor são analisados e debatidos.

Palavras – chave: Maneio reprodutivo, Reprodução, Elefantes, Zoos, Conservação.

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List of Abbreviations and Symbols

% - Percentage
°C – Degrees Celsius
3D – Three-dimensional reconstruction
acCL – Accessory CL
AI – Artificial Insemination
Al – Allantois
Am – Amnion
anLH – Anovulatory LH peak
AV – Artificial vagina
Av – Allantoic vessels or arteria vertebralis
AZA – Association of Zoos and Aquariums
BC – Before Christ
BIAZA - British & Irish Association of Zoos and Aquariums
bpm – Beats per minute
CITES – Convention on International Trade in Endangered Species of Wild Fauna and Flora
CL – Corpora Lutea or Corpus Luteum
cm – Centimetres
DM – Dry matter
Dr – Doctor
e.g. – exempli gratia
Ea – Ear
EAZA – European Association of Zoos and Aquariums
EAZWV – European Association of Zoo and Wildlife Veterinarians
ECZM – European College of Zoological Medicine
EEHV - Elephant Endotheliotropic Herpesvirus
EEP – European Endangered species Programmes
EIA – Enzymimmunoassay
ELISA – Enzyme-linked immunosorbent assay
Em – Embryo
Ev – Embryonic vesicle
Ey – Eyes
Fl – Front legs
FSH – Follicle-stimulating Hormone
GnRH – Gonadotropin-releasing hormone
GnRH-Ag – Gonadotropin-releasing hormone Agonist
h – Hour(s)
He – Heart
Hl – Hind legs
iCa – Ionized serum calcium
ICSI – Intra-Cytoplasmatic Sperm Injection
Ir- Inhibin – Immunoreactive Inhibin
IUCN – International Union for Conservation of Nature
IV – Intravenous
IVF – In Vitro Fertilisation
IZW – Leibniz Institute for Zoo and Wildlife Research
kg – Kilogram
L – Liter(s)
LH – Luteinizing Hormone
mg – Milligram
mg/kg – Milligram per kilogram
Mh – Midgut herniation
min – Minute(s)
mmol/L – Millimolar per liter

nm – Nanometers
ng/mL – Nanograms per millilitre
ovCL – Ovulatory CL
ovLH – Ovulatory LH peak
PhD – Philosophiae Doctor
Pl – Placenta
PRL – Prolactin
RIA – Radio Immuno Assay
SSP – Species Survival Plan
T3 – Triiodothyronine
T4 – Tiroxina
TAG – Taxon Advisory Group
Tr – Trunk
TSH – Thyroid-stimulating hormone
Ul – Uterine lumen
US – Ultrasound
Uv –Umbilical vessels
VLDL – Very light-density lipoprotein
x – Time(s)
Ys – Yolk sac
Z 7-12:Ac – *Z*-7-dodecenyl acetate
µm – Micrometer

Internship activities

Training activities report

The curricular internship was accomplished at the Leibniz Institute for Zoo and Wildlife research (IZW) in Berlin. I had the opportunity to participate in various veterinary activities and side projects which involved the handling of animals kept at the facility. The practical part with a duration of 8 months, from September 2013 until May 2014, comprised 800 hours.

During that period, my main focus was to gain a deeper understanding of how reproductive management aids conservation efforts in captive elephants. In this context, under the guidance of Prof Dr Thomas Hildebrandt, I was able to assist four veterinary reproductive examinations, which will be further detailed in this work. With all the preparations and travels to the Zoos, around 50 hours were spent in these assessments.

Regarding elephants, I participated also in one non-reproductive examinations performed at Leipzig Zoo (German), in which I could improve my knowledge about endoscopy technique, during a trunk exploration to collect bronchial lavage fluid, to be further tested for tuberculosis.

Besides elephants, I was able to assist other veterinary reproductive assessments in a variety of species (around 50 hours for clinical activities in other zoo):

- Ultrasonography of male and female gorillas and chimpanzees, during which the practical tasks included opening an intravenous route, monitoring anaesthesia and collection of blood samples, guided by Prof Dr Thomas Hildebrandt,
- Ultrasonography of hyenas for gender determination and collection of saliva and hair samples, performed by Prof Dr Thomas Hildebrandt,
- Ultrasonography and artificial insemination of two female white rhinos, thereby assisting with the processing of frozen semen samples, under the supervision of Dr Robert Hermes,
- Ultrasonography and electroejaculation for semen collection of a wolf, learning the main important steps to achieve a good sample for cryopreservation with Dr Robert Hermes and Dr Frank Göritz.

Furthermore, I was able to follow the routine activities performed by the veterinarians at the IZW, and to participate in numerous projects during the course of the internship. These activities included assisting the daily management of animals kept and bred at the institute for research purposes, for example guinea pigs and naked mole rats, in which I spent most of my internship time (around 350 hours), since the infrastructures are allocated at IZW. Some of the common tasks consisted in feeding, health checks and application of simple treatments, temperature monitoring and weighting. In the case of naked mole rats, I performed behavioural observations regarding their social hierarchy, and assisted in ultrasound examinations, micro-chipping, semen collections with the use of electroejaculation, tissue sampling for genetic testing and necropsy procedures.

In guinea pigs, research involved recording of ultrasonographic images of embryonic and foetal development during several phases of pregnancy. After learning the technique and milestones with Dr Susanne Holtze, I was able to independently perform the examinations in the pregnant females. Further, I performed the castration of one male guinea-pig, under supervision.

The IZW holds a field station (Niederfinow, Germany), which houses European roe deer for scientific purposes, mainly regarding the reproductive system. There, I participated in the capture, anaesthesia (by blow darting) and monitoring, endotracheal intubation, and sample collection, and further observed the ultrasonographic assessments of the reproductive status, as well as testicular biopsies. Vaccinations, the monitoring of post-vaccination immune reactions, as well as tissue and hair sampling were also performed in hares at this place. I was for several weeks at this field station, comprising around 200 hours of my curricular practical teaching.

During my internship I also assisted researchers from other departments in smaller tasks. In a PhD project that involved collaring wild boars for GPS tracking, I collected samples (hair and blood), measured temperature and monitored anaesthesia under the supervision of Dr Frank Göritz. For a PhD-project on bat metabolism, I learned how to perform brown fat biopsies in hibernating bats. I spent around 50 hours in other projects training activities.

Organizing and packing the travel equipment needed for examinations of the “Department of Reproduction Management” abroad, transmitted a great knowledge of the available and necessary veterinary equipment and the essential steps of various reproductive techniques performed in many wildlife species; e.g., tigers, rhinos, elephants, chimpanzees, gorillas, bears, etc.

I was also able to acquire more profound knowledge and to assist in Computed Tomography examinations of live and dead animals, which are regularly performed at the research institute.

Moreover, blood collection and subsequent processing to obtain serum or plasma samples, as well as biochemical and haematological analyses were frequently performed as part of my work at the department’s laboratory.

In 2014, I participated in the International Conference on Diseases of Zoo and Wildlife Animals in Warsaw, Poland, which is an annual event where European zoo veterinarians and wildlife researchers meet, present and debate interesting clinical cases and new discoveries in this area.

Although the gross amount of cases seen was in the field of reproduction management, the experience of contacting with diverse and interesting species, the literature research regarding the cases at hand and the newly acquired clinical knowledge added considerable value to this practical curricular internship. A total amount of 100 hours was spent on research activities during the masters' internship.

Chapter1 Introduction

1.1 Use of elephants by man

The interaction between humans and elephants started long ago, during the Stone Age, when elephants were being hunted alongside their extinct relatives, the mammoths. Elephants appear in art work dating back to 30.000 BC in Europe, and also in Neolithic paintings in Africa (approximately 4.500 - 2.000 BC). However, the earliest evidence of elephants in captivity starts only at around 4.500 years ago, long after evidence of most other livestock species (10.000 BC, Csuti, 2006).

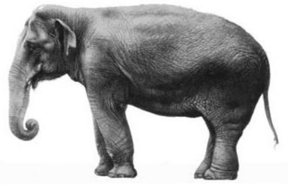
Since then, elephants have been used as war machines by several empires on different continents and were considered to have had an important influence on the course of several battles (Sukumar, 2003). Elephants also served as valuable offers for many European Kings, and Portugal was no exception; “Hanno”, an Asian white elephant was offered to Pope Leo X at his coronation by King Manuel I of Portugal. Hanno arrived in Rome in 1514 and became the Pope's favourite animal, astounding the papal court and being the star of many processions, festivals, paintings, and sculptures for the Roman people. Nowadays, ceremonial elephants are still present in human culture, especially in India, alongside with working elephants, which are used for their capacity of working in steep logging terrain as well as for tourist entertainment in Africa and Asia. The first evidence of the exhibition of elephants in circuses dates back to the ancient Indian princes and to the Romans (Csuti, 2006).

1.2 Taxonomy

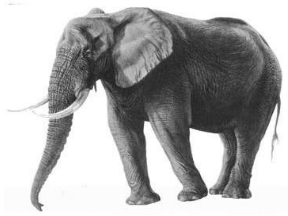
The elephant belongs to the Kingdom Animalia, Phylum Chordata, Class Mammalia and Order Proboscidea, which contains only one extant family - the Elephantidae, including the generae *Loxodonta,* with two described species (Rohland et al., 2010) and *Elephas*, with four extant subspecies (Blanc, 2008) (Table 1).

Table 1: Common name and scientific name of the existent elephant species and subspecies. (Adapted from Blanc, 2008; Rohland et al., 2010; AZA, 2011). Representative image of *E. maximus* (top) and *L. Africana* (below) for comparison (Images source: British & Irish Association of Zoos and Aquariums (BIAZA), 2010).

Common name	Scientific name
Asian elephant	*Elephas maximus (E.maximus)*
Indian elephant	*E. m. indicus*
Sri Lankan elephant	*E. m. maximus*
Sumatran elephant	*E. m. sumatranus*
Bornean elephant	*E. m. borneensis*
African Savanna Elephant	*Loxodonta africana (L.africana)*
African Forest elephant	*Loxodonta cyclotis (L.cyclotis)*



Elephas maximus



Loxodanta africana

1.3 Current status context of African elephant

In this thesis, I have mainly focused on the African elephant as this is the species currently maintained in captivity at Lisbon Zoo. However, analogies with the closely related Asian elephant are made use of whenever literature is lacking for *Loxodonta.*

The African elephant was classified in 1986 as Vulnerable by the International Union for Conservation of Nature (IUCN), maintaining this status until 1996, when it acquired the Endangered classification. In 2004, this species was again rated as Vulnerable by the IUCN Red List of Threatened Species, and has been keeping this category until today. At the moment, the main threat faced by the African elephant is poaching for ivory and meat, constituting the major cause for the species' decline. The increase of human-elephant conflicts further aggravates the threat to elephant populations within their distribution range. *Loxodonta africana* are now regionally extinct from Burundi, Gambia and Mauritania, and were reintroduced to Swaziland (Blanc, 2008).

The Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) listed the African elephant as one of the most endangered species, threatened with extinction. It "prohibits international trade of all specimens of these species "except when the purpose of the import is not commercial (…) for instance for scientific research". In these exceptional cases, trade may take place, "provided it is authorized by the granting of both an import and an export permit". These regulations exclude the African elephant populations in Botswana, Namibia, Zimbabwe and South Africa, countries in which the species is considered to be at a lower risk of extinction (http://cites.org).

1.4 Zoos, conservation and major concerns

Elephants have been kept in royal animal collections for at least 3,500 years. One of the most famous examples is the menagerie at Versailles (1665 - 1681). Also Schönbrunn Zoo, in Vienna, once an imperial collection, is the world's oldest zoo, and has been keeping elephants since 1752 (wwww.zoovienna.at).

According to the European Association of Zoos and Aquaria (EAZA), a new strategic Masterplan for European Endangered species Programmes (EEP) which was released in 2013, the captive African elephant population is considered to be in a highly critical situation. The captive birth rate increased over the years; however numbers are still not sufficient to counterbalance the death rate (Schwammer & Fruehwirth, 2013). Likewise, the population contains a large group of females between their mid-twenties to early thirties, which is expected to cause a peak in mortality in the future. Also, the majority of these females are considered not to be able to breed again (Hermes et al., 2004; Schwammer & Fruehwirth, 2013), while proven breeders constitute the minority of the EEP population (Table 2).

Table 2: Demographic distribution for the African Elephant EEP population, regarding their breeding population status on February 15th of 2013; (information from Schwammer & Fruehwirth, 2013).

	Females	Males	Total
Current EEP population	139	46	185
Excluded from breeding population	60	3	63
***Potential breeders**	79	43	122
****Proven breeders**	25	8	33

* Animals at breeding age
**Individuals from the potential breeding group already confirmed as breeders after reproductive examination

Therefore, reproduction management of captive elephants becomes one of the major conservation efforts that zoos can provide to create self-sustaining populations of African elephants, both under human care and in the wild.

1.5 New aid technologies

Captive elephant groups constitute highly fragmented isolated populations. As such, inbreeding and the consequential loss of genetic diversity and fitness (including reproductive fitness) of the population are of constant concern regarding population management (Hermes et al., 2009).

Recent advances in technologies and methodologies have allowed for a better understanding of the reproductive biology of elephants. Through the use of ultrasound examinations, for example, Hildebrandt (2000a, 2000b) and his team were able to identify morphological abnormalities and to reveal the importance of this non-invasive method for identifying potential breeders that are healthy and suitable for natural or assisted breeding efforts. Therefore, among the captive elephant population, ultrasound examinations are considered to be an essential tool by the Association of Zoos and Aquariums (AZA) elephant Species Survival Plan (SSP) and EAZA studbook breeding programme (Schwammer & Fruehwirth, 2013).

Likewise, further methodologies concerning the development of sperm cryopreservation or the study of hormonal cycles will advance our knowledge of assisted reproduction in elephants. Routine endocrine monitoring of the oestrous cycle is achieved through sampling of faeces, blood, and urine and is nowadays considered an indispensable tool for the successful management of elephant breeding groups in captivity (Hildebrandt et al., 2012).

1.6 Main Goals

This thesis aims at reviewing the current knowledge on the African elephant reproductive biology and management. For this purpose, I have collected bibliographic information comprising publications of the last fifteen years, compiled to a comprehensive literature review.

The main goal is to present the latest discoveries, empirical results, as well as, methodologies employed to study reproduction in elephants, and to assemble this information as a consultation manual for orientation within the management facilities. Therefore, I have structured this thesis into seven relevant chapters:

Chapter I – Introduction of the general situation of captive elephants and the importance of this dissertation;

Chapter II - Reproductive biology of both female and male elephants, spanning anatomy, physiology and associated pathologies;

Chapter III - Management techniques, including semen collection, as well as hormonal contraception and estrous cycle induction;

Chapter IV - Pregnancy and parturition, including management techniques of the gestating cow and known cases of dystocia;

Chapter V - Neonatal care in captivity and the most important milestones and interventions during the first months of the calves;

Chapter VI - African elephant population at the Lisbon Zoo, focussing on the current breeding status of the herd and reproduction management recommendations;

Chapter VII – Clinical cases assisted by the author, to enhance understanding on the conservation efforts being done from a reproductive point of view to aid the elephant population.

Chapter 2 Reproductive Biology

2.1 Female elephant

2.1.1 Reproductive Anatomy of the Female

Female elephants present unique reproductive characteristics. For many years, these constituted a challenge and an obstacle for breeding programs, given that techniques developed for domestic or laboratory species were not applicable to this mega vertebrate mammal. Concerning anatomy, techniques for organ visualisation by rectal ultrasonography had to be developed, including the design of a suitable probe extension (Figure 3), which is normally required to examine the most cranial portions of the 3 m long urogenital tract of the female elephant (Hildebrandt et al. 2006) (Figure 1 and 2).

Figure 1: Schematic representation of the female elephant reproductive tract (image source:http://www.asianelephantresearch.com/about-elephant-anatomy-and-biology-p4.php).

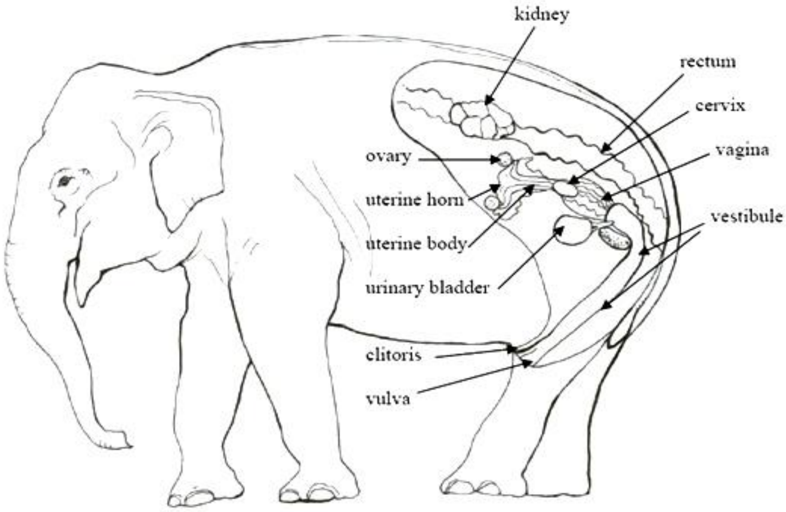


Figure 2: Illustration of the female urogenital tract: ovaries (OV); uterus bicornis (UT); cervix (CE); vagina (VA); vaginal os (VO) with two bilateral blind pouches; vestibule (VE); urinary bladder (UB); urethra (UR); pelvic bone (PB) (modified from Hildebrandt et al., 2006).

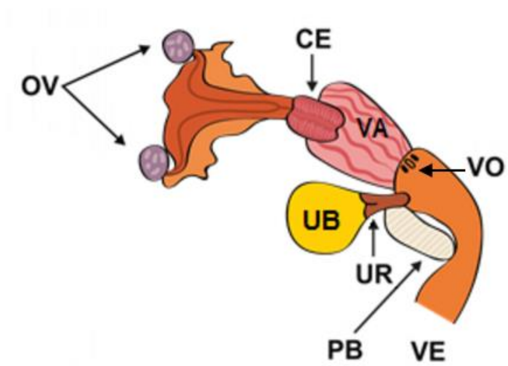


Figure 3: Example of extension used for US examination; adapting the US probe to the extremity of the extension allows the operator to scan deeper organs. Video glasses to monitor the ultrasound exam are shown on the bottom (Courtesy of Dr Thomas Hildebrandt, Department of Reproduction Management – Leibniz Institute for Zoo and Wildlife Research (IZW), Germany).

Vestibule

Female elephants possess a vestibule, or *canalis urogenitalis*, which is positioned ventrally between the hind legs, opens at the vulva, and has a length of 1 – 1.4 m. It is a tube-like structure, which runs vertically up to underneath the anus, where it curves cranially. At this point, it acquires a horizontal position, running near the pelvic bone (Schmitt, 2006; Lueders & Hildebrandt, 2012). There it creates a sac (20 – 40 cm) and meets the urethra (with a transurethral fold) and the vaginal opening (Hildebrandt et al., 2000a; Schmitt, 2006) (Figure 4).

The *glans clitoris* (7 – 12 cm) is located close to the external opening of the vestibule, protruding from it during urination and relaxation (Hildebrandt et al., 2000a). The clitoris continues through the vestibule, being integrated into the muscular wall of the vertical part and believed to help directing the penis into the vestibular canal (Hildebrandt et al., 2000a; 2012).

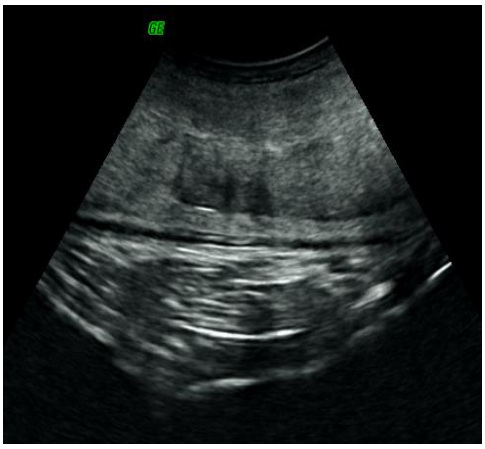


Figure 4: Ultrasound image of the vestibule in cross section (Courtesy of Dr Thomas Hildebrandt, Department of Reproduction Management – IZW, Germany).

Vagina

In nulliparous females, a hymen-like structure is found at the cranial end of the vestibule, leaving only a small orifice of approximately 0.4 × 0.2 cm to the vagina. (Hildebrandt et al., 2011; 2012; Lueders & Hildebrandt, 2012; Wiedner, 2015).This opening at the hymeneal membrane is flanked by two blind pouches, thought to be the vestigial remains of the Wolffian ducts (Hildebrandt et al., 2000a).

The penis is introduced only into the cranial part of the urogenital canal during copulation, and not directly into the vagina. Therefore, this structure will not rupture during mating, but only during the first parturition (Hildebrandt et al., 2011), after which the diameter of the vaginal orifice increases to approximately 2 - 3 cm in diameter (Lueders & Hildebrandt, 2012; Wiedner, 2015). Hildebrandt et al. (2000a) reports that one year postpartum the opening measures around 1 x 1 cm and the blind pouches disappear.

The vagina possesses longitudinal folds and measures approximately 30 x 15 x 10 cm, (Hildebrandt et al., 2000a; Wiedner, 2015). It may extend to a total length of 50 cm (Hildebrandt et al., 2011; 2012) and contains mucous. The accumulation of mucous varies according to the phase of the estrous cycle (Hildebrandt et al., 2000a) (Figure 5). The amount of mucous increases markedly during the transition from luteal to follicular phase, and gradually dissolves between the two Luteinizing Hormone (LH) peaks, being excreted during urination (Hildebrandt et al., 2011). This mixture of urine and mucous contains specific sexual pheromones, which for breeding bulls serves as an attractant towards receptive females (Rasmussen et al., 2008). During pregnancy the vaginal mucous increases in thickness, functioning as a protective barrier against mechanical damage and infectious agents (Hildebrandt et al., 2000a; Schmitt, 2006).

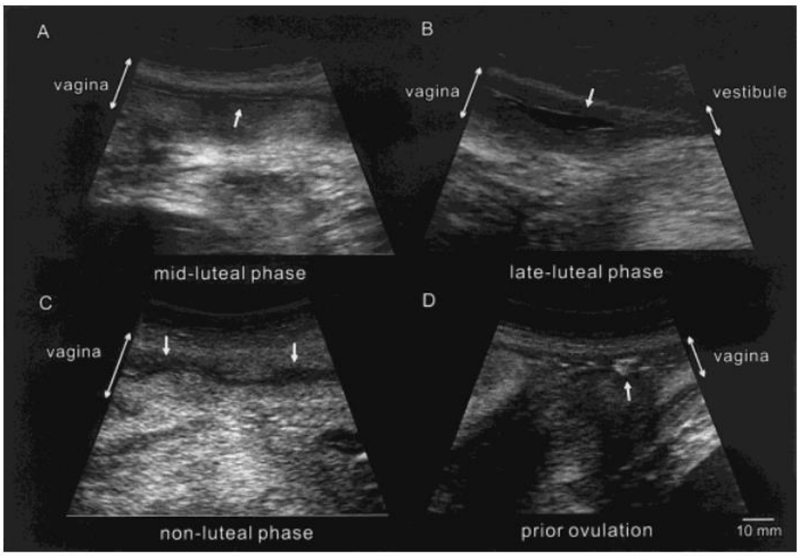
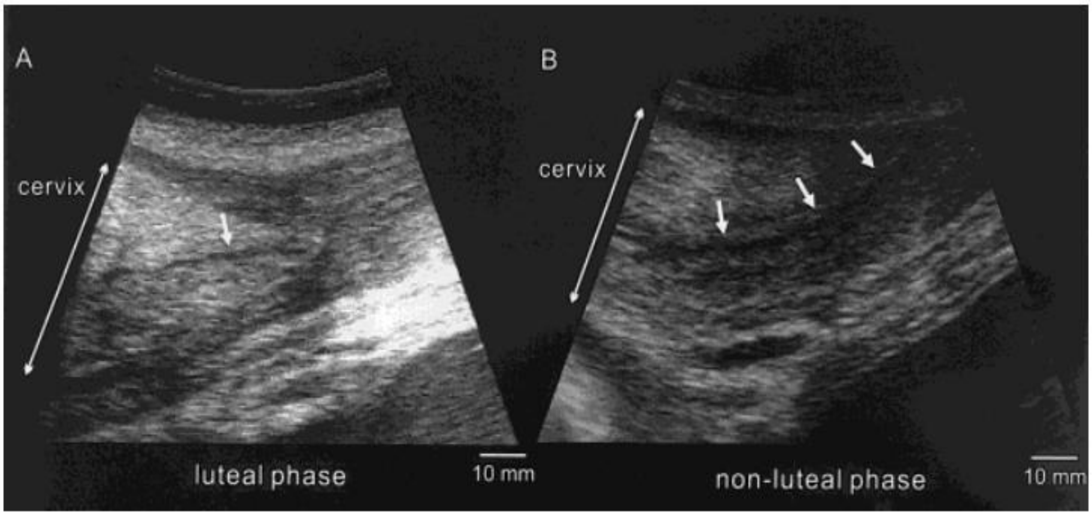


Figure 5: Ultrasound images in longitudinal view of the vagina in an African elephant, at different stages of the oestrous cycle: A) Mid-luteal phase with small mucous volume (arrow); B) Late luteal phase, showing a large volume of vaginal mucus (arrow); C) Follicular phase phase, with a reduced echogenicity of the vaginal mucosa (arrows) and D) Before ovulation, with echodense vaginal mucus, showing a broken, shell-like appearance (arrow) (Source: Hermes et al., 2000).

Cervix

The cervix exhibits longitudinal folds (as found in the mare) and measures approximately 15 cm in length. It holds a prominent cone-shaped *portio cervicalis* (Hildebrandt et al., 2011; Lueders & Hildebrandt, 2012) of approximately 9 x 7 x 5 cm that protrudes into the vagina, resembling a rosebud (Hildebrandt et al., 2000a; 2012; Schmitt, 2006). Cervical mucosa thickness ranges from 8 - 15 mm (Hildebrandt et al., 2000a). The cervical canal can also be visualized via sonography; its aspect changes during the different stages (Hermes, 2000). During luteal phase this structure appears less echogenic, while during follicular phase it is well distinguished as an anechogenic band (Figure 6).

Figure 6: Ultrasound images of the cervix in an African elephant (in longitudinal view) (Source: Hermes et al., 2000). A – Luteal phase: the cervix presents a triangular shape and central cervical canal is less echogenic (arrow) B – Nonluteal phase: the cervical canal is well distinguished as an anechogenic band (arrows).



Uterus

The bicorneal uterus measures between 0.8 – 1.5 m in length. It comprises a short uterine body, *corpus uteri*, of 5 – 10 cm (Lueders & Hildebrandt, 2012) and long uterine horns of approximately 50 – 70 cm with a lumen of about 12 – 45 mm (Hildebrandt et al., 2006).

The endometrial lining of the uterine body and horns presents longitudinal *rugae* (Schmitt, 2006) and both possess a ciliated mucous membrane (Lueders & Hildebrandt, 2012). Pronounced changes in the appearance of the endometrium in ultrasound examinations occur over the course of the estrous cycle (Figure 7) (Hermes et al., 2000; Hildebrandt et al., 2006). Endometrial thickness is variable (Figures 8, 9 and 10); the uterine body has a thinner endometrial lining, which may explain why implantation does not occur in this structure, but only in the uterine horns (Lueders & Hildebrandt, 2012). Hildebrandt et al., (2000a) mention that the phenomenon of twin pregnancy in elephants may rarely occur due to the wide separation between the two horns. In such cases, calves are delivered independently, with a long inter-birth interval (up to several months), with neither offspring being adversely affected.

With each pregnancy, damage to the endometrium occurs where the placenta has been attached, leaving permanent circularly shaped scars (Allen et al., 2003). These scars can be detected during post-mortem or ultrasound examinations, and indicate the number of previous pregnancies in animals of unknown reproductive history (Hildebrandt et al., 2006). Additionally, according to their appearance, the time interval between the last birth and a sonography examination can be estimated (Hildebrandt et al., 2000a).

Figure 7: Ultrasound images of the uterus in an African elephant (longitudinal view). Endometrium height measures by the two-headed arrow; A) Before ovulation: the low echogenic endometrium is at its maximum height and fluid is observed in the uterus; B) Four days after ovulation: the endometrium is more echodense and although still large in height, becomes difficult to distinguish from the myometrium. Fluid is observed in the uterine lumen; C) Luteal phase: characteristic flat endometrium; D) Non-luteal phase: the endometrium is less echogenic and increased in height. Endometrium and myometrium are well distinguished and blood vessels are observed in the myometrium. The central white line indicates adjacent endometrial folds (Source: Hermes et al., 2000).

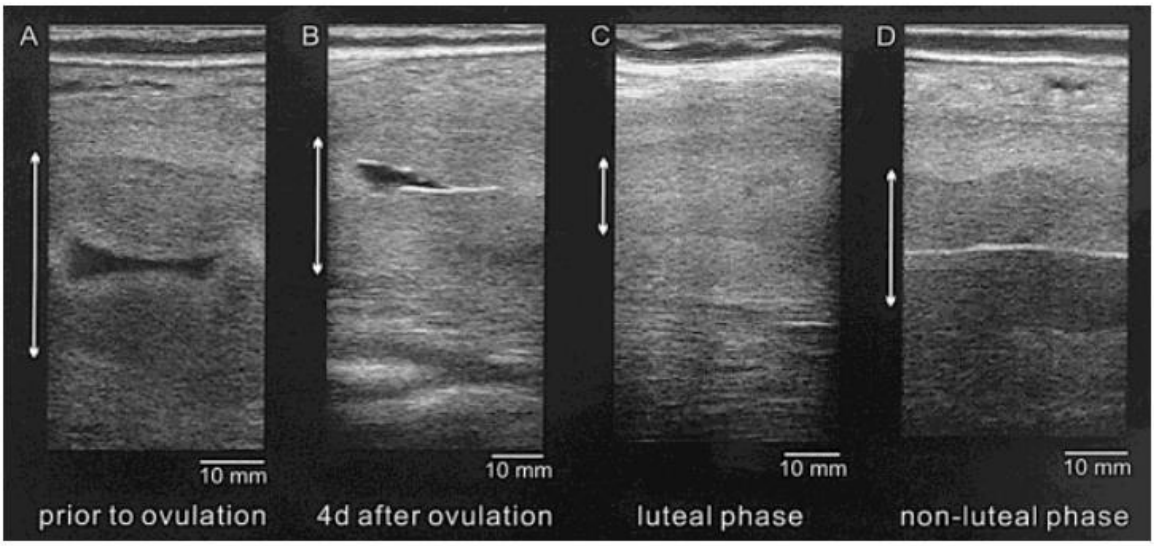


Figure 8: Ultrasound image of the uterus in early follicular phase (Courtesy of Dr. Thomas Hildebrandt, Department of Reproduction Management – IZW, Germany).

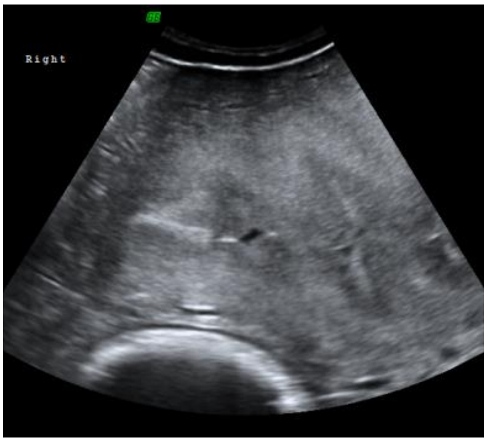


Figure 9: Ultrasound image of the uterus horn, presenting thickened endometrium and mucous content in the lumen near ovulation (Courtesy of Dr Thomas Hildebrandt, Department of Reproduction Management – IZW, Germany).

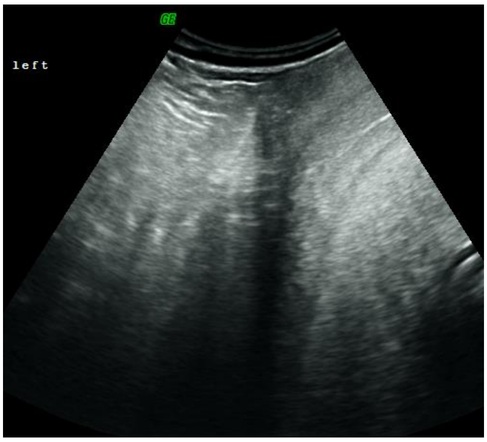


Figure 10: Ultrasound image of the uterus (longitudinal view), during luteal phase (Courtesy of Dr Thomas Hildebrandt, Department of Reproduction Management – IZW, Germany).

Oviducts

The oviducts are approximately 10 cm long (Schmitt, 2006) and their mucosa appears hypoechoic when compared to the surrounding tissue. In ultrasound examinations, the oviducts are considered important landmarks for locating the ovaries within the abdominal cavity (Hildebrandt et al., 2000a) (Figure 11).

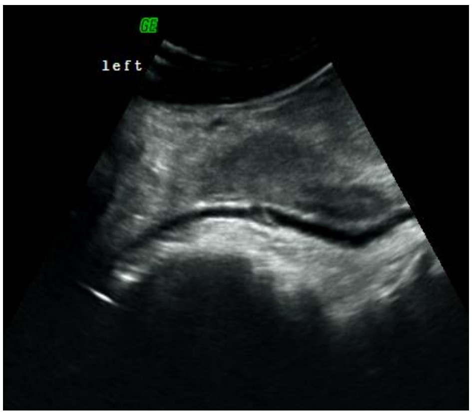


Figure 11: Ultrasound image showing a partial view of the coaled oviduct (longitudinal view) (Courtesy of Dr Thomas Hildebrandt, Department of Reproduction Management – IZW, Germany).

Ovaries

At three to four years of age, the ovaries develop a convoluted, brain-like surface, which is detectable by ultrasonography (Hildebrandt et al., 2000a). The ovaries are completely enveloped in a double pouch. The inner serosal pouch forms the oviductal infundibulum and the external pouch encapsulates the ovary. Additional fat inclusions can complicate the ultrasound examination of the ovarian structures (Lueders & Hildebrandt, 2012). In juveniles, the ovary is divided into the echoic central *medulla ovarii* and a less echogenic peripheral *cortex ovarii* (Hildebrandt et al., 2000a). Until puberty, no large follicles or *corpora lutea* (CL) are visible on the ovaries (Schmitt, 2006).

The ovarian size of an adult female elephant is small. Ovaries weigh approximately 60 g and measure ca. 7 x 5.5 x 2.5 cm^3 (Lueders & Hildebrandt, 2012) (Figure 12). For healthy females, the ovary always presents functional structures, except for during the late lactational anestrus (Lueders & Hildebrandt, 2012).

Lueders et al. (2010) measured the size of the dominant follicle on the day of ovulation, in a study conducted on 11 Asian female elephants and has found that, on average, mature follicles measure 20.2 ± 0.8 mm, are of round shape with anechoic appearance, and a typical white line can be seen below these fluid-filled structures (Hildebrandt et al., 2000a) (Figure 13).

Figure 12: Ultrasound image of a dormant ovary (left side) although presenting normal size, in early follicular phase (Courtesy of Dr Thomas Hildebrandt, Department of Reproduction Management – IZW, Germany).

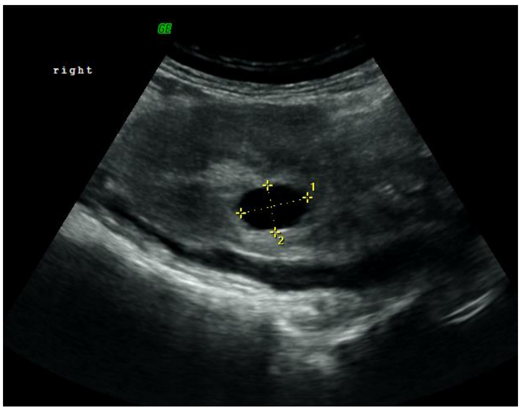


Figure 13: Ultrasound image of the ovary (right side) with a visible Graafian follicle in development (1.62 x 1.10 cm) (Courtesy of Dr Thomas Hildebrandt, Department of Reproduction Management – IZW, Germany).

An interesting feature of elephants is the development of multiple *corpora lutea* (on average 4 – 6 and a reported maximum of 42), measuring between 10 - 50 mm. These appear as a protrusion from the ovarian cortex in cycling as well as in pregnant cows (Lueders & Hildebrandt, 2012). Two different types of CL can be found in the elephants: accessory CL (acCL) and the ovulatory CL (ovCL). Both types are moderately echogenic, sometimes with an elongated echogenic center and a homogeneous parenchyma. The acCL appears fluid-filled and differs from the ovCL in shape, location within the ovary and the absence of stigmata (Hildebrandt et al., 2000a) (Figure 14).

In ultrasound examinations, around 2 - 10 acCL can be found on each ovary of a cycling or pregnant female elephant (Lueders & Hildebrandt, 2012). It is proposed that these acCL will help to maintain a (future) pregnancy (Lueders et al., 2012).

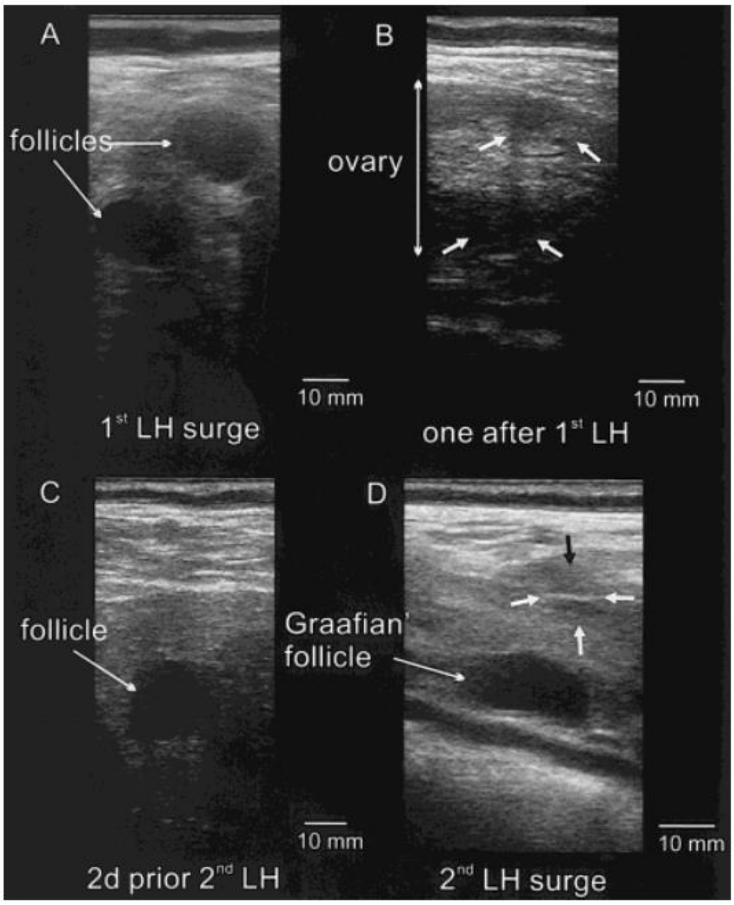


Figure 14: Ultrasound images of the ovary in an African elephant. A) Two follicles are visualized in luteinisation process, already starting to produce a wall and leading to the formation of an acCL (see below); B) One day after the first anLH surge; luteinisation process of the previously imaged follicles (arrows); C) Dominant follicle in growing process, before the second ovulatory LH (ovLH) surge; D) Graafian follicle on the day of ovLH surge and a CL in formation (arrows) (Image source: Hermes et al., 2000).

2.1.2 Physiology of the oestrous cycle

Female elephants present poliestric spontaneous ovulation and are considered non-seasonal breeders. Although peaks of births have been observed in the wild (Lueders & Hildebrandt, 2012) this phenomenon has yet not been reported in a more stable captive environment. There are however, some differences between the responsiveness of Asian and African elephants: Brown (2014) states that Asian elephants seem to show more consistent cycles, even when facing management changes, in contrary to African elephant females, for which temporary or permanent suppression of the oestrous cycle has been

repeatedly reported (e.g., after translocations, changes in blood collection frequency, changes in herd dynamics or exchange of keepers).
Elephants possess the longest oestrous cycle known among all mammals, lasting 13 - 17 weeks. It includes a luteal phase of 8 - 10 weeks and a 4 - 7 weeks inter-luteal, i.e. follicular period (Brown, 2014). Therefore, captive females may express only three to four fertile cycles per year (Hildebrandt et al., 2011). The oestrous cycle length may vary between individuals but seems to be extremely consistent for each individual cow (Brown, 2000). Synchronisation of oestrous cycles has been reported by Weissenböck et al. (2009) in a captive group of four African female elephants, and similar observations have been made in human care (T. Hildebrandt, personal communication, 2014).
Wild elephants are continuous breeders, and cycles with no conception seem to be rare, as females are usually found either gestating or lactating (Hildebrandt et al., 2006). Unfortunately, this is not the reality for most captive elephants.

Luteinizing Hormone, Progestagens, Inhibins, and Follicle-stimulating Hormone

The remarkable difference of elephants' oestrous is the occurrence of two luteinizing hormone (LH) peaks, with comparable concentrations and separated by 3 weeks, during the follicular phase. Each LH peak coincides with a follicular wave. The first LH surge constitutes the anovulatory peak (anLH), and the second the ovulatory peak (ovLH) (Lueders et al., 2010). At the first anLH surge only follicles that presented with a diameter larger than 7.5 mm are luteinized. This occurs ca. 7 ± 2.4 days before the second LH peak and serves to create the acCL. The follicles smaller than 7.5 mm consequently start regressing (Lueders et al., 2010; 2011).
Simultaneously with the luteinization of this first wave of follicles, immunoreactive inhibin (ir-inhibin) concentrations rise and stay elevated for 41.8 ± 5.8 days after ovulation (Lueders et al., 2011). Another finding is that inhibin α, βA and βB subunits are imunnolocalised in both granulosa cells of antral follicles and luteal cells in the ovary. These findings strongly suggest that the corpus luteum is one of the sources of inhibin, as well as the follicles themselves (demonstrated in an Asian elephant, Kaewmanee et al., 2011). As a consequence, the luteinized follicles should be of importance in the selection of the dominant follicle (Lueders et al., 2011). The concentrations of ir-inhibin return to baseline 20-35 days before the end of the luteal phase and it starts to decline after the acCL have reached their maximum size (Lueders et al., 2011). The serum ir-inhibin levels are positively correlated with progesterone throughout the oestrous cycle and negatively correlated with Follicle-stimulating Hormone (FSH) during the late non-luteal and early luteal phases (Kaewmanee et al., 2011). There is still an elevation in FSH-levels during the luteal phase, even with high inhibin concentrations, suggesting that inhibin alone is not capable of suppressing FSH completely (Lueders et al., 2011). The inverse relationship between FSH

and inhibin concentration provides further evidence of the important role of inhibin secretion in dominant follicle selection (Lueders et al., 2011; Yamamoto et al., 2012).

As ovulation always occurs on the ovary with higher amount acCLs, and given that follicles lutenize before the dominant follicle reaches its ovulatory dimensions, Lueders et al. (2010; 2011) suggest that the luteinized follicles predetermine which ovary will ovulate. Therefore, inhibins might act locally on the ovary itself, besides their influence on FSH secretion.

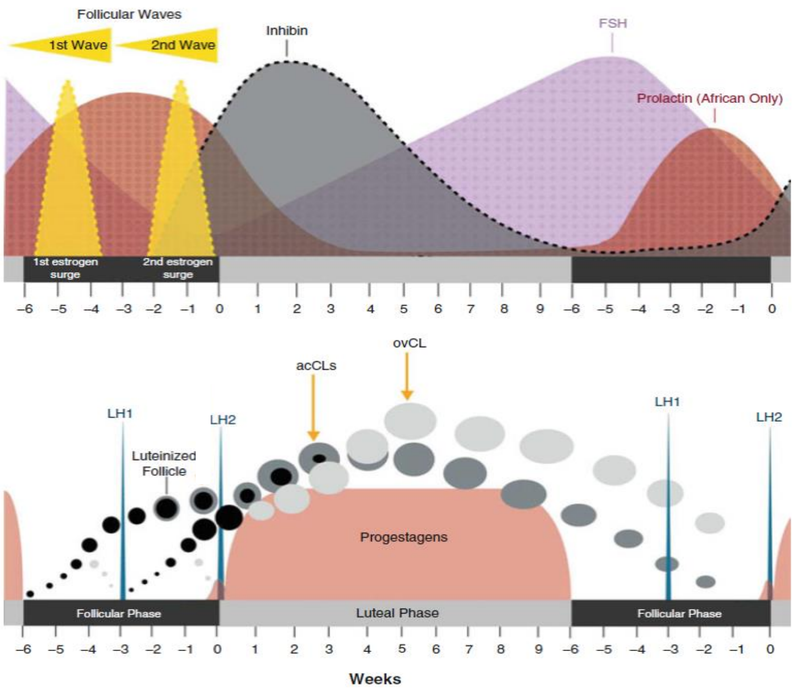
Brown et al. (2004) studied and compared the endocrinology of the estrous cycle between African and Asian elephants. They report that both species present a cyclic pattern of FSH secretion: its concentration is highest at the beginning of the follicular phase, drops to baseline levels four days before the second LH surge, remaining low until after this peak, and finally increases during the luteal phase. The FSH-peak at the beginning of the follicular phase seems to impel folliculogenesis on the removal of the progesterone block (when levels drop to a minimum) (Lueders et al., 2010; Lueders & Hildebrandt, 2012).

From the second follicular wave, a single dominant follicle is created, ovulating 12 to 24 hours after reaching around 20.0 mm in diameter (Hildebrand et al., 2000a; Lueders et al., 2010) (Figure 15). The ovCL can be distinguished by ultrasonography from ovarian tissue 7-10 days after ovulation, and continues to increase in diameter until 35 – 42 days of the luteal phase (Lueders et al., 2011).

Unlike the majority of mammalian species, the main progestagen secreted by the CL is not progesterone, but its 5α-reduced metabolites or pregnanes (e.g., 5α-pregnane-3,20-dione, 5α-pregnane-3-ol-20-one, 17α-hydroxyprogesterone), referred to as progestogens (Brown, 2014).

Lueders et al., (2011) reported that while the progestagen metabolite was elevated there was no follicular growth, but as soon as it reached baseline values follicles started developing. The progestagen levels rise rapidly, 1 – 3 days after ovulation, reaching a maximum of 3,7 ng/mL in the fifth week of the luteal phase. Therefore, it is probable that the second LH peak is important to mature the luteal cells of the acCL and makes them steroidogenically active. This initial progestogen secretion may be produced by these accessory acCLs, since they present already a more advanced stage; acCLs reach the maximum diameter within 30 days after ovulation, while the ovCL becomes significantly larger 10–15 days later (Lueders et al. 2010). This means that the luteal regression of the acCL occurs while the ovCL continues to grow or maintains its size for a few more days. The decline in progestagen concentration (functional luteolysis) is followed by a reduction in the diameter of the ovCL (structural luteolysis). At this moment, all CLs are still surprisingly large (Lueders et al., 2010), and the absence progestogens despite the presence of luteal tissue remains an intriguing question (Lueders, 2011).

Figure 15: Scheme of the ovarian cycle, representing hormones secreted during the follicular and lutheal phase (estrogen, progestogens, LH, FSH, prolactin and inhibin). The development of the two follicular waves culminate at the 1st and 2nd LH peak and the formation of follicles, leading to the two types of *corpora lutea:* ovulatory and accessory CL. In this image prolactin curve is presented only for the African elephant, since the Asian species is not influenced by any stage of the cycle (Adapted from Brown, 2014).



Prolactin

There is a major difference in prolactin secretion between the two elephant species (Hildebrandt et al., 2012). For the Asian elephant, the concentrations of prolactin remain stable throughout the entire estrous cycle, while African elephants present the highest prolactin concentration during the follicular phase which is inversely related with progestagen secretion. Abnormal production of prolactin (hyperprolactinemia or the lack of cyclic secretion) is commonly linked with acyclicity in African elephants (Brown et al., 2004a).

Oestrogens

One of the most important oestrogens during the estrous cycle is estradiol. Estradiol serum concentration varies during the cycle, averaging less than 25 pg/mL with occasional peaks up to 65 pg/mL. There are also increases in estradiol preceding the LH surges (Brown et al., 2004a).

2.1.3 Endocrine monitoring

Endocrine monitoring is the key to assessing reproductive status in elephants, facilitating breeding efforts. It can easily be accomplished by measuring the concentration of different hormones using a variety of assay techniques (Brown, 2006). The most accurate results are obtained by using a Radio Immuno Assay (RIA). Unfortunately, not many laboratories use this technique, and therefore, will not be able to provide a daily service. Tests that are based on other immunological reactions – enzyme immune assay (EIA) and enzyme linked immunosorbent assay (ELISA) – are routinely used in human hospitals, but need to be validated for elephants before the results can be trusted (Schaftenaar & Hildebrandt, 2005).

Profiles may be generated using serum, plasma, urine and faeces. Whenever possible, endocrine analyses should begin as soon as an animal tolerates blood collection and this sampling can be incorporated into the routine management program. When this is not possible, an available option is to monitor gonadal status non-invasively, through the analysis of faecal and urinary steroid metabolites (Brown, 2000). Urine analysis requires additional processing steps, i.e. the measurement of creatinine to account for variation in fluid intake, and the hydrolysis of steroid conjugates to liberate free steroids to be assed.

Fecal samples are easier to collect, but analyses are more laborious and the process is more expensive. Another negative aspect is the lack of a suitable index (such as creatinine) to standardise the results. Also, a comparatively long excretion lag time and the difficulty of collection of an appropriate sample are critical in faeces analysis, as the steroid concentrations are not evenly distributed (Brown, 2000).

The hormone profile must be assessed as soon as possible, during the entire reproductive life of a dam. The most important hormones are summarized below:

Progestogens: Progestogens can be measured in blood, and its metabolites in urine and faeces. Serum samples for assessing progestogens are recommended monthly for initial evaluation, increasing to weekly intervals as the female reaches an age of 4 or 5 years and during early gestation. From week 89 - 91 (637 days) daily sampling is recommended, so collaboration with a laboratory that can run the samples daily must be established.

Prolactin (PRL): PRL can be measured only in blood (Brown., 2000). Serum samples for PRL assays are recommended; sampling frequency and sensitivity of the RIA have been validated by Yamamoto (Yamamoto et al., 2012).

Cortisol: Cortisol can be measured in blood, and its metabolites in urine, saliva and faeces. However, the evaluation of cortisol in urine appears to be a better indicator of its activity (Brown, 2000).

Oestrogens: Oestrogens can be measured in blood and urine. Faecal oestrogen concentration does not reflect serum concentration.

LH and FSH: These hormones are only detectable in the blood. Either no secretions of native LH can be detected in urine, or the concentrations present are too low to be measured by the standard immunoassay techniques used for serum analyses in both Asian and African elephants (Brown et al., 2010).

2.1.4 Pathology of the female reproductive tract

In the past, nulliparous females with 15 years of non-reproductive periods were not considered problematic. Based on their long reproductive life-span in the wild (Hermes et al., 2004), animals with 30 years of age or more were considered potential breeders by the breeding programs. However, there is clear evidence in African and Asian female elephants, that older cows (especially nulliparous ones older than 30 years of age) exhibit increased incidence of pathological alterations that develop throughout the reproductive tract (Hildebrandt et al., 2000a). Often, these pathologies are not suspected because there is continuation of regular ovarian cycles and the cows exhibit no other clinical signs of reproductive problems. Non-reproducing cycling females present a shortened reproductive life-span (15 years earlier, in extreme cases) when compared with reproducing females. This suggests that the continuous cyclicity of non-bred females may have a negative and cumulative effect on their reproductive health. In the wild, females are either pregnant or lactating, and therefore, they experience comparatively fewer reproductive cycles in their lifetime (Brown, 2004b) and reproductive tumours or cystic hyperplasia are almost non-existent.

The premature senescence of captive female elephants is due to an asymmetric reproductive ageing process, better explained by the effect of long-term exposure to sex steroids through continuous ovarian cycle activity, which can negatively affect the reproductive organs of these non-reproducing females. This seems to cause a progressive development of genital pathologies, leading to reduced fertility. In order to prevent this asymmetric reproductive ageing process, establishing pregnancy in young animals seems to be imperative (Hermes et al., 2004).

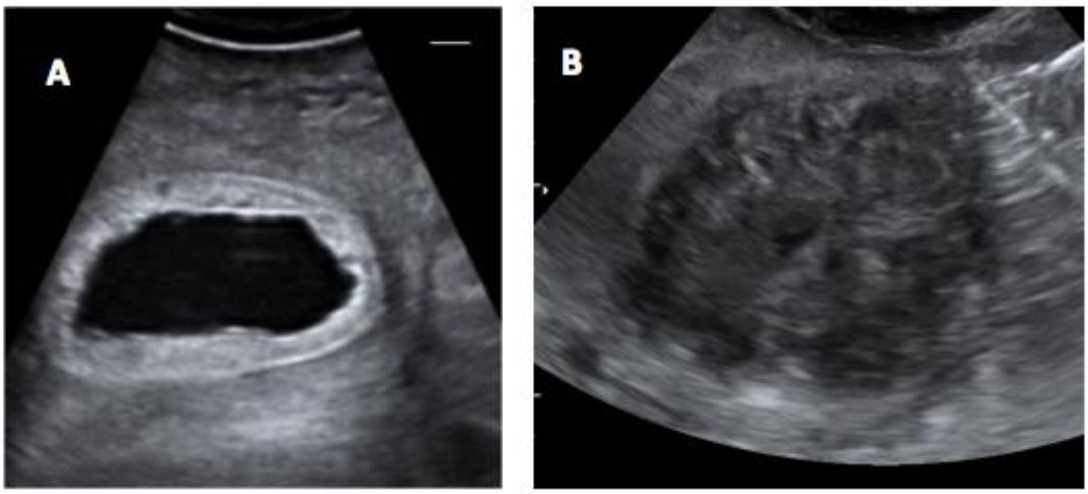
With ultrasound and endoscopy most of these morphological abnormalities are easily identified and their impact on fertility can be investigated (Lueders & Hildebrandt, 2012) (Figure 16). Ultrasonography examinations should be conducted in potential breeding candidates exhibiting no history of pregnancy or long inter-birth intervals (Hildebrandt et al. 2000a). Treatments for most of the pathologies of the reproductive tract are not reported and only early breeding may reduce their likelihood of occurrence. They are not fatal and some do not necessarily cause pregnancy failure. The outcome depends on location and severity of these pathologies (Lueders & Hildebrandt, 2012).

Another effect associated with non-bred captive cows is acyclicity. Acyclicity affects mostly Asian elephants older than 30 years, while in African elephants it can be found in all adult age groups (Schmitt, 2006; Lueders & Hildebrandt, 2012). This state can be maintained permanently or temporarily. Temporary non-reproductive acycling status in elephants seems to actually protect them from the asymmetric ageing process; since the absence of circulating ovarian hormones seems to prevent the development of steroid-dependent tumours or cystic endometrial hyperplasia. Elephants with late puberty or long periods of temporary acyclicity show less incidence of genital pathologies, similar to what is observed in early pregnancy cases.

There are various reasons for the establishment of irregular or total absence of cyclic activity: seasonal changes (e.g., time spent indoors); distress-activated high cortisol levels (e.g., oestrous cycle cessation due to translocation); social factors such as hierarchy status (e.g., dominant African elephant females are reported to be more likely to be acyclic) (Lueders & Hildebrandt, 2012).

Finally, another commonly observed female pathology is infertility. In a study conducted by Hildebrandt et al. (2000a), several Asian females that started cycling by the age of four years were unable to conceive at ages of 14 – 19 years despite observed mating. Hildebrand (2000c) states that female infertility occurs temporarily or permanently in captivity and is always linked to changes of the internal genital organs, based on the results of reproductive assessments of 280 females and 75 bulls.

Figure 16: Examples of pathological uterine findings during ultrasound in female Asian elephant. A: Pyometra in the uterine horn; fluid content in the uterine horn (black) and inflamed endometrium (white); B: Leiomyoma in uterine horn with destruction of normal appearance; no distinction of myometrium and endometrium is possible (Source: Luerdes & Oerke, 2016).



In the following paragraphs, the most commons pathologies associated with the reproductive tract are listed.

Vulva and Vestibule

Cysts – Vestibular cysts are frequently found in captive elephants of both species (Hildebrandt et al., 2000a).

Polyps – In contrast to the cystic lesions, vestibular polyps, up to 5 cm in diameter, occur only in older captive African cows (approximately 70% of African elephant females older than 30 years of age). These alterations apparently have no direct impact on reproductive health; however, they are clear indicators of advanced age (Hildebrandt et al., 2000a).

Scars – Vestibular scars can be found in the pelvic rim region. These alterations in the vestibular mucosa and/or muscle may have originated from different causes such as urogenital infection, mechanical injuries during mating or birth, episiotomy for artificial insemination, or injury from the incorrect use of an elephant hook. Females may experience discomfort due to the presence of scars, as they sometimes react strongly to rectal and vestibular palpation, or avoid natural mating (Hildebrandt et al., 2000a).

Vulvitis – Lympho-follicular vulvitis of unknown cause or clinical significance is sometimes observed, that consists of hyperaemic nodules or plaques in the mucosa of the distal urogenital tract (Stevenson & Walter, 2006).

Papillomas and sarcomas have also been reported in the distal urogenital tract of both female elephant species. Urogenital discharges are relatively common, resolving without treatment in many cases (Stevenson & Walter, 2006).

Urinary tract

Although rare, urinary bladder infections may occur in elephants, predominantly in old or immunodeficient cows. Although with no direct influence on the ability of the cow to conceive, these alterations may become extensive and painful, and the female will not allow a bull to mate (Hildebrandt et al., 2000a).

Vagina

For both species, nulliparous cows older than 30 years of age present frequently with cysts in the vaginal mucosa. Although these cystic alterations may have no direct impact on conception, they might be an indicator of progressive aging in addition to other pathological alterations of the upper genital tract. In two reported cases, vaginal cysts became so extensive they filled the entire vaginal lumen (Hildebrandt et al., 2000a).

Cervix

The main cervical pathology found in both elephant species is cystic lesions, which occur more frequently in older captive African and Asian elephants (approximately 15% of those examined) than in wild African females (< 1%). Occasionally, small cervical polyps with a maximal diameter of 1 cm were detected in African cows. There was one case of scarring in the cervical tissue, caused by rupture during parturition. Another rare birth-associated alteration in an Asian cow involving the cervix and the caudal part of the uterus was a permanent subcutaneous prolapse (large bulb under the tail) caused by partial rupture of the genital ligament. The affected individuals were more sensitive to the rectal palpation procedure. Female elephants trained too young to perform behaviours that result in non-physiological abdominal contraction also tend to develop pelvic prolapses. The pelvic diaphragm is not yet strong enough to accommodate the abdominal pressure and the genital tissue becomes compressed into a bulge under the anus that can cause parturition problems later in life (Hildebrandt et al., 2000a).

Uterus

Both species exhibit markedly different uterine pathologies; Asian elephants have a tendency to develop multiple benign uterine tumors in the myometrium (leiomyomas) after a limited non-fertile reproductive period (10–15 years), while African elephants have not been observed with these neoformations, but rather tend to develop a cystic endometrium (endometrial hyperplasia) (Hermes et al., 2004). A less common finding, sometimes observed in captivity, but rarely in the wild (~2%), is a resorbed embryo or early foetus that resulted in pyometra, presented as a non-infectious, pus-filled cavity, at the end of the uterine horn (Hildebrandt et al., 2000a).

Oviducts

African cows were reported of having cystic lesions in this region. Paraovarian cysts reaching up to 5 cm in diameter have no effect on the reproductive cycle, and may be misinterpreted as ovarian structures (Hildebrandt et al., 2000a).

Ovaries

Minor pathologies of the ovary such as follicular cysts can be found in both species. Small cysts originating in follicular structures are located in the outer cortex, while pre-Graafian follicles are found in the parenchyma. Ovarian cysts are reported to affect ~5% of captive Asian and ~15% of captive African elephants. However, they are rarely observed in free-ranging African females (<1%) (Hildebrandt et al., 2000a).

2.2 Male elephant

2.2.1 Reproductive Anatomy of the Male

For a better anatomical understanding, Figure 17 and 18 present a schematic and illustration representation of the male reproductive tract.

Figure 17: The male elephant reproductive system schematic representation (image source: http://www.asianelephantresearch.com/about-elephant-anatomy-and-biology-p4.php).

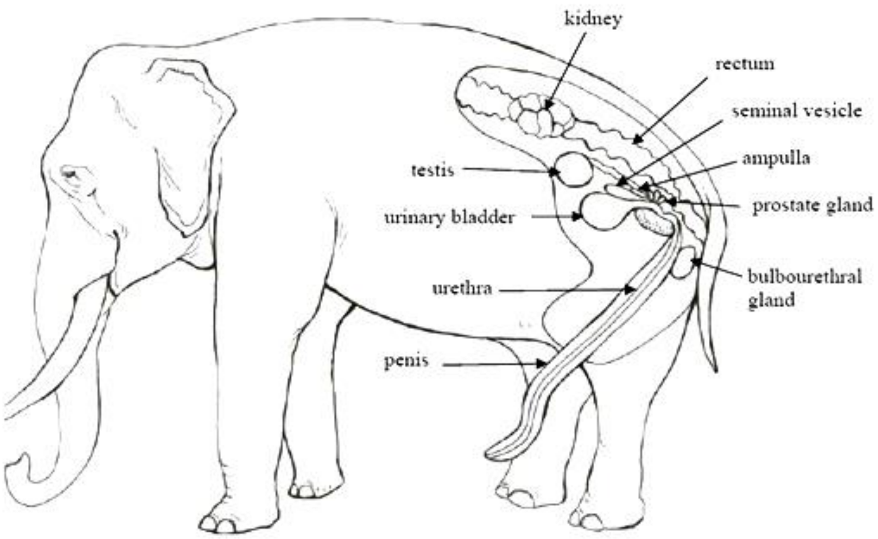
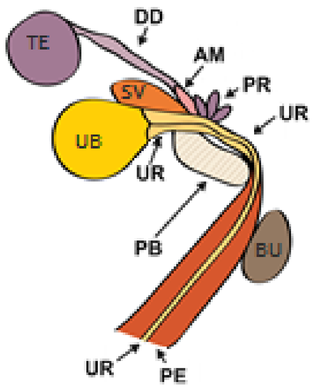


Figure 18: Illustration of the main parts of the urogenital tract: testis (TE); ductus deferens (DD); ampulla (AM); seminal vesicle SV); prostate (PR); bulbo-urethral gland (BU); urinary bladder UB); urethra (UR); penis (PE); pelvic bone (PB), modified from Hildebrandt et al. (2006).



Penis

The adult bull presents a heavily vascularized penis, with a weight that can reach over 27 kg, an approximate length of 100 cm (Moss et al., 2011) and a diameter of approximately 20 cm (Hildebrandt et al., 2000b). It possesses a well-developed *corpus cavernosum* (Woodford et al., 2000). Experts still have not reached a conclusion on whether the elephant bull possesses a true prepuce or penis glands (Schmitt, 2006).

Most of the central urethra can be visualized by transcutaneous ultrasonography. The penis complex associated Cowper's muscle is reported to form a bulge under the tail in a few individuals. Generally, this bulge becomes more prominent during musth, but there is no correlation between the size of the bulge and fertility (Hildebrandt et al., 2000b).

During erection the penis acquires an S-shaped curve due to the contraction of the paired large levator penis muscles on its dorsal surface (Woodford et al., 2000), thereby elevating the vulva into position prior to penetration (Hildebrandt et al., 2006). During mating, the penis presents a high mobility, allowing movements independent of the pelvis and therefore, little pelvic thrusting or movement is needed from the male to achieve an intromission and ejaculation (Schmitt, 2006).

Bulbourethral Glands

The bulbourethral glands present a fist size and are situated about 2 cm underneath the skin surface, immediately caudal to the root of the penis (Hildebrandt et al., 2000b).

On the ultrasound examination these accessory paired glands appear as primarily solid tissue with a central irregularly shaped cavity filled with secretion when visualized before ejaculation. The bulbourethral glands are large and produce a viscous secretion (Woodford et al., 2000). They contain a clear and sticky content that ranges from 5 to 20 mL per pair. These glands are believed to be involved in cleansing and pH-balancing of the urethral environment, and to support penis emission in the female vestibule during copulation (Hildebrandt et al., 2000b) (Figure 19).

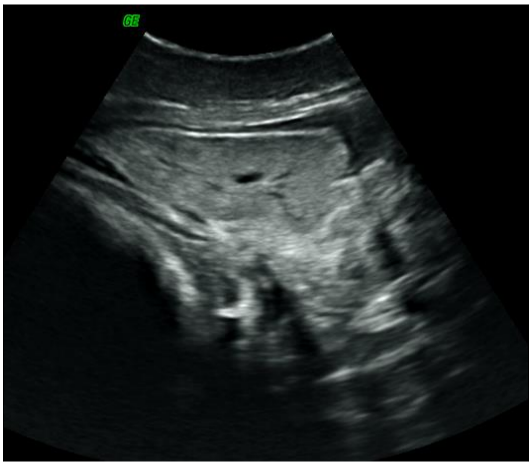


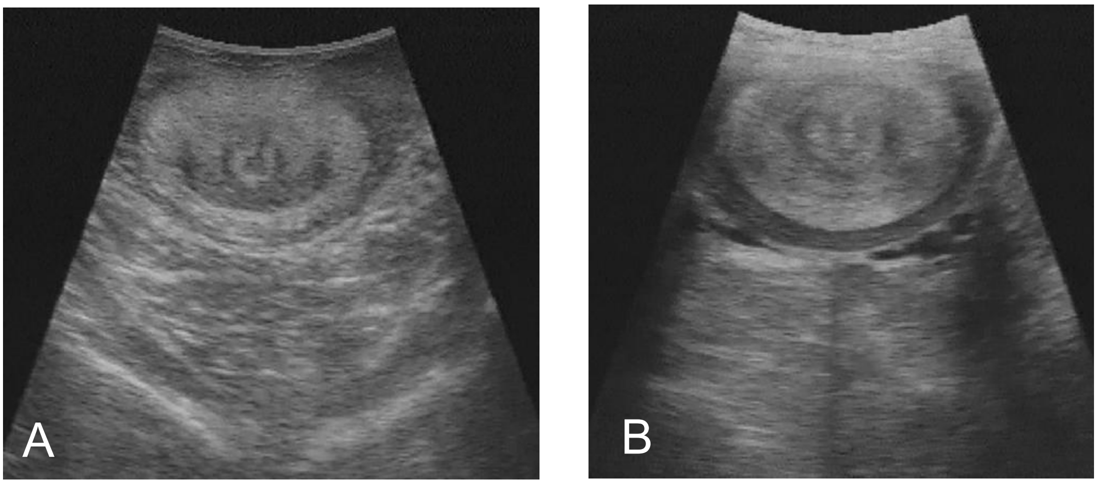
Figure 19: Ultrasound image of the bulbourethral gland (left side), with irregular cavity (Courtesy of Dr Thomas Hildebrandt, Department of Reproduction Management – IZW, Germany).

Urethra

The urethra in both female and male elephants is a well-defined structure with a strong internal sphincter. In males, the total length is on average 2.0 m with a fully erected penis, and it has a diameter ranging from 35-55 mm. (Hildebrandt, 2006) (Figure 20).

The pelvic urethra in the male differs from the female's only by the presence of the *colliculus seminalis* (located about 1 cm caudal to the opening of the urinary bladder), that is an integral part of the dorsal urethral wall, approximately 1.5 cm long and 8 mm in diameter. This structure contains the joint ducts of the *ampullae*, the paired seminal vesicles, and several individual ducts (at least three from each side) from the prostate lobes. These ducts have a maximal diameter of 1 mm and can be imaged inside the colliculus by using high-frequency transducers, but cannot be distinguished from each other (Hildebrandt, 2000b).

Figure 20: Ultrasound images of the urethra in a male African elephant; A – penial aspect of the urethra, with corpus cavernosum, B – Urethra in the pelvic cavity (Courtesy of Dr Thomas Hildebrandt, Department of Reproduction Management – IZW, Germany).



Prostate Glands

A pair of prostate glands is located on the dorsal wall of the urethra, immediately posterior to the seminal vesicles (Woodford et al., 2000). Each pair possess three lobes joined by an isthmus, and can be found bilateral to the pelvic urethra and caudally to the *ampullae*. The prostate is the only accessory gland that varies in size and composition between the two elephant species (Hildebrandt et al., 2000b; Hildebrandt, 2006b). The African elephant presents a larger prostate (maximum 5 cm in diameter) with irregular-shaped internal cavities and prominent glandular tissue present in each lobe (Figure 21 and 22), which are absent in the Asian elephant. For the Asian elephants, the prostate shows a 2 cm diameter with largely homogeneous tissue (Schmitt, 2006).

In the previously cited study, Hildebrandt et al., (2000b) found no obvious characteristics of the adult prostate that could be used as indicators of fertility. However, they found a dramatic developmental difference in size and complexity of the glands between immature and adult males, which could be used as indicators of fertility.

Figure 21: Ultrasound image of an inactive prostate (right side) of an African elephant. Due to the absent internal cavities it has a similar aspect as normal functional prostate of Asian elephant males (Courtesy of Dr Thomas Hildebrandt, Department of Reproduction Management – IZW, Germany).

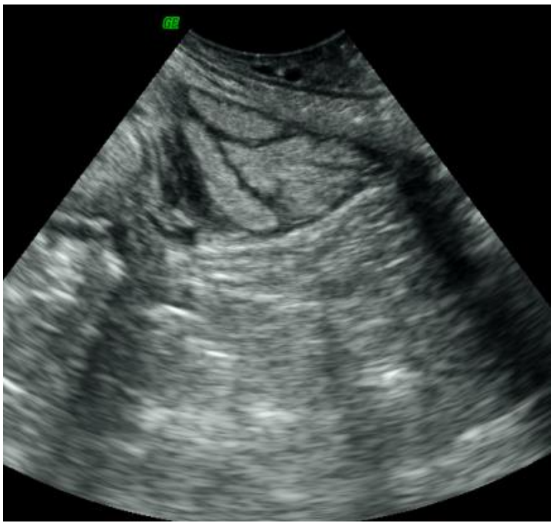
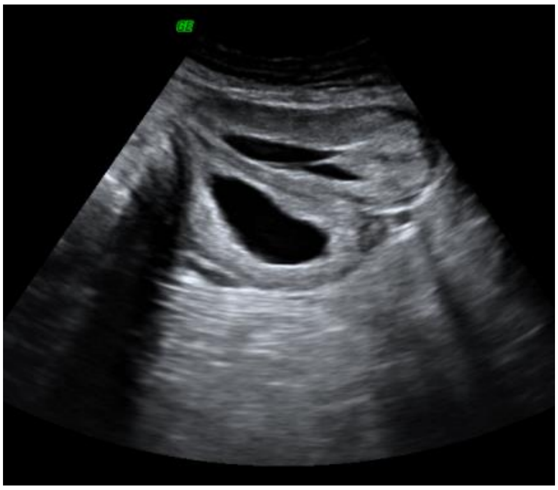


Figure 22: Ultrasound image of the prostate presenting irregular-shaped internal cavities in an African elephant (Courtesy of Dr Thomas Hildebrandt, Department of Reproduction Management – IZW, Germany).



Ampullae

As male elephants do not possess well-developed epididymis like most mammals, spermatozoa are stored inside of the *ampullae* – a cone shaped structures (Hildebrandt et al., 2000b). Each *ampulla* presents a maximum diameter of 5 cm, measuring 6 – 8 cm in length and it is located on the terminal part of the ductus deferens, above the urinary bladder at the cranial part of the urethra. The circumference of the *ampullae* (measured in cross-section) presents their filling state, and the echogenicity of the fluid indicates the sperm concentration. In few bulls flat *ampullae* could be seen on US examination and their ejaculates were nearly aspermic. Therefore, the *ampullae* can be used as a predictor of the amount of sperm obtainable through manual semen collection, making these glands one of the most important structures to assess during ultrasound evaluations of testicular activity.

However, this parameter does not correlate always to quality of the ejaculates; in a study performed by Hildebrandt et al. (2000b), the majority of the mature bulls presented highly echogenic and well-expanded *ampulla*e, but the ejaculate presented mostly immotile sperm.
In immature bulls the *ampullae* present an undeveloped status, and in these animal's semen collections are mainly unsuccessful or produce small amounts of ejaculate with immature sperm cells (Hildebrandt et al., 2000b) (Figure 23 – 27).

Figure 23: Ultrasound image of the *ampulla* (left) before ejaculation (Courtesy of Dr Thomas Hildebrandt, Department of Reproduction Management – IZW, Germany).

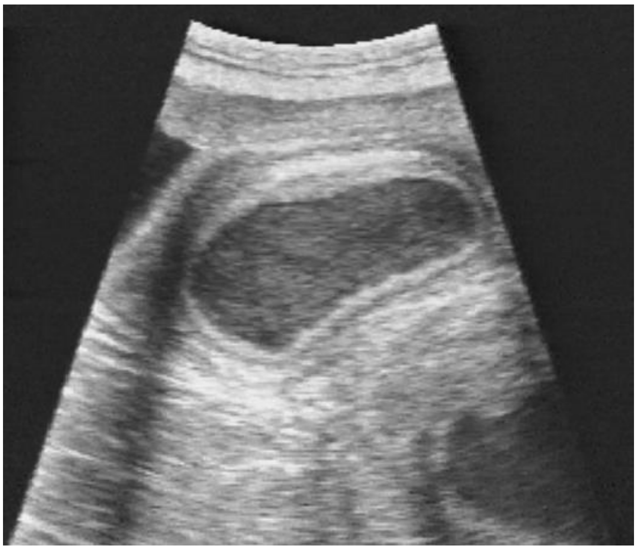


Figure 24: Ultrasound image of the *ampulla* (left) after ejaculation (Courtesy of Dr Thomas Hildebrandt, Department of Reproduction Management – IZW, Germany).

Figure 25: Ultrasound images of the left and right *ampullae* showing different densities; image on the right presents higher spermatozoa concentration (can concentrate over 1 billion sperm cells per millilitre) (Courtesy of Dr Thomas Hildebrandt, Department of Reproduction Management – IZW, Germany).

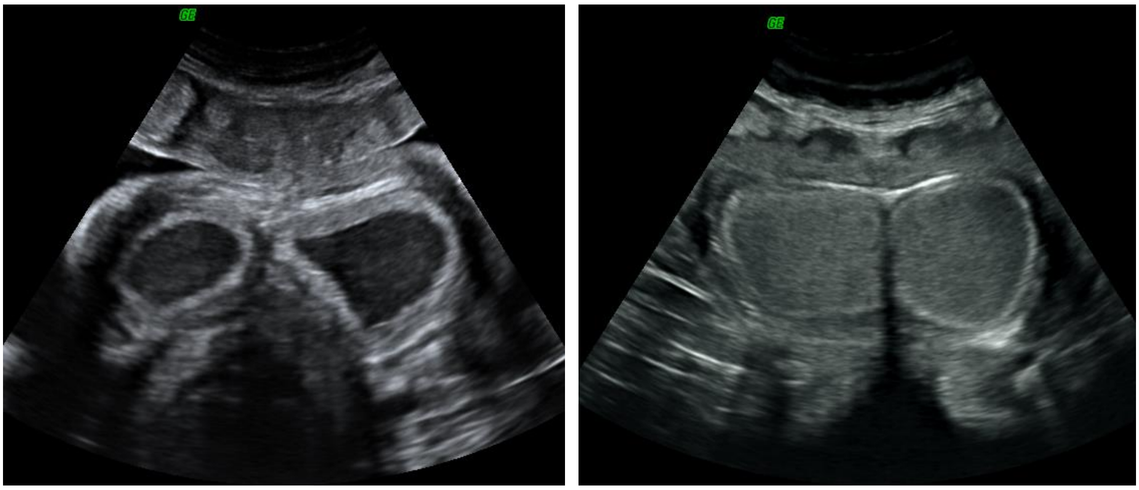


Figure 26: Ultrasound image of an *ampulla* (left side) presenting two different densities, that is a sign of long lasting storage, leading to a gravity based separation, with the death of sperm cells (Courtesy of Dr Thomas Hildebrandt, Department of Reproduction Management – IZW, Germany).

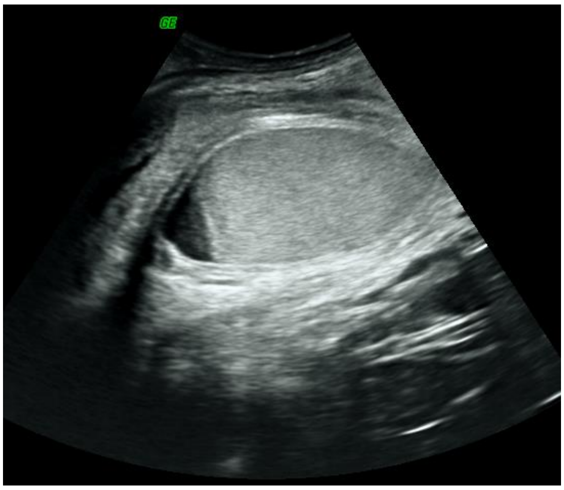
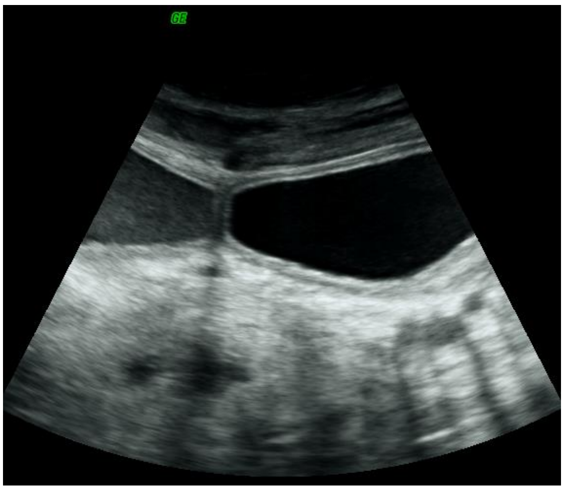


Figure 27: Ultrasound image of the *ampullae* showing different echogenicity: *ampulla* presented on the right side contains a anechoic fluid, due to non-functional right testicle which is a very unusual situation (Courtesy of Dr Thomas Hildebrandt, Department of Reproduction Management – IZW Germany).



Seminal vesicles

Each seminal vesicle can contain up to 400 mL of fluid, therefore making it the largest accessory gland of an active breeding bull. Seminal vesicles are characterized by a single cavity with a 5 – 10 mm thick wall, composed of an outer muscular layer and an internal mucosa that may be clearly distinguished on US examination (Figure 28). These paired glands produce the majority of the seminal fluid, providing nutrients and a vehicle for the sperm. Due to their great variability, in combination with the *ampullae* these glands are the most effective factors to characterize a bull's breeding potential (Hildebrandt et al., 2000b) (Figure 29).

Figure 28: Ultrasound image of the left seminal vesicle (Courtesy of Dr Thomas Hildebrandt, Department of Reproduction Management – IZW, Germany).

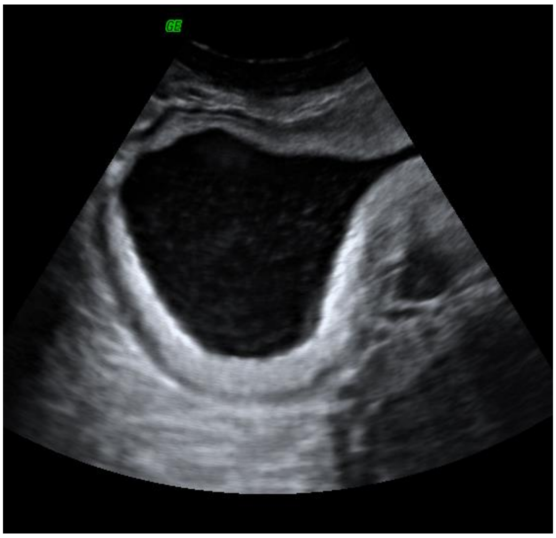
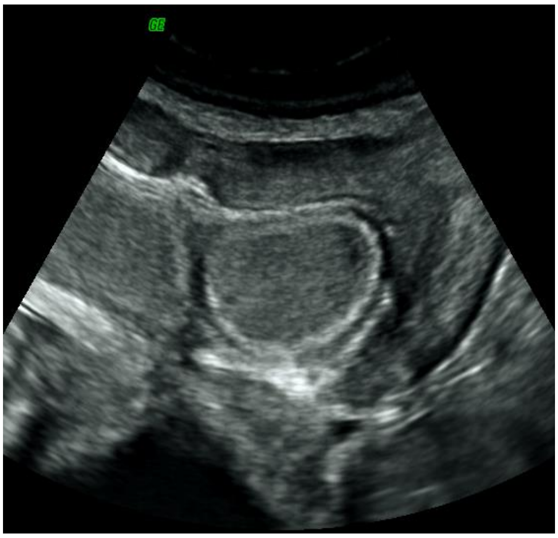


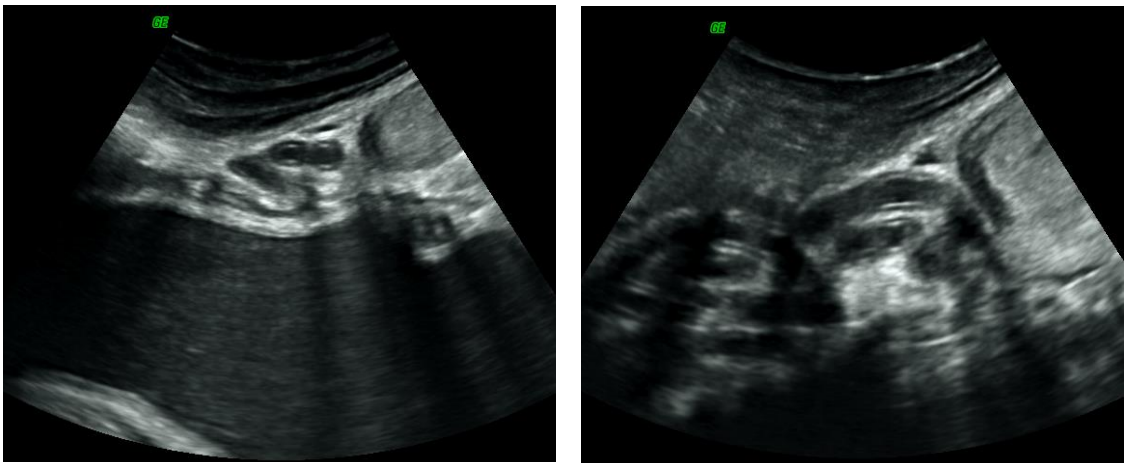
Figure 29: Ultrasound image of the *ampullae* and seminal vesicle (left side) (Courtesy of Dr Thomas Hildebrandt, Department of Reproduction Management – IZW, Germany).



Ductus Deferens

The ductus deferens complex is strongly coiled, measuring approximately 1 m in length and up to 2 cm in diameter, although the total length cannot be visualized in ultrasound examinations (Figure 30). In adult bulls the ductus deferens range from 5 - 10 mm in diameter. The cranial part (adjacent to the testis), is only visualized using an extension for the ultrasound probe, but the straight caudal part (located between the seminal vesicle and the urinary bladder) is easily assessed using a hand-held probe. In active breeding bulls, the caudal part contains an anechoic lumen, indicating presence of sperm, while in castrated males it is nearly undetectable (Hildebrandt et al., 2000b).

Figure 30: Ultrasound image of the ductus deferens with visible lumen (Courtesy of Dr Thomas Hildebrandt, Department of Reproduction Management – IZW, Germany).



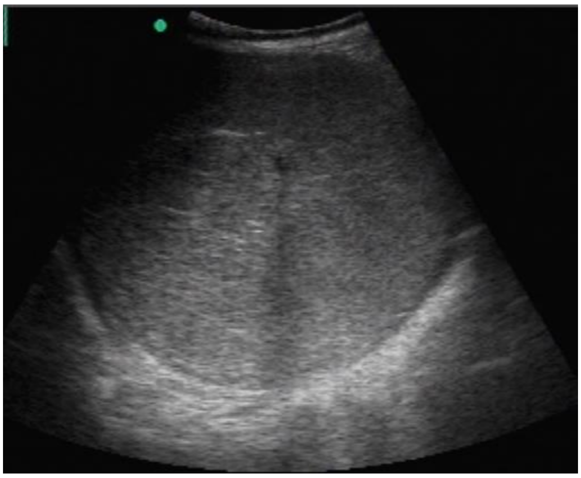
Testes

The testes of an elephant are retained intra-abdominally and situated caudal to the kidneys. Although a pampiniform plexus and the cremaster muscles are absent (Woodford et al., 2000), elephants still manage to maintain an intra-testicular temperature of 34º - 36º, the same that is found in scrotal testes of most other mammals (Hildebrandt et al., 2006).

There is a single testicular artery penetrating the testis by the hilus that radiates into smaller arteries. This pattern contrasts with scrotal testes, where the testicular arteries penetrate the parenchyma from its surface. The testes show size variation during ontogenesis: they measure on average 2 cm in newborns, and ca. 9 cm in the prepubertal animal. During these developmental stages the major blood vessels are embedded in the mediastinum testis. It is reported that only testis bigger than 10 cm, can produce sperm-rich ejaculates (Hildebrandt et al., 2000b). In adults the testes can reach a weight up to 1.5 kg (Woodford et al., 2000) (Figure 31).

Hildebrandt et al. (2000b) evidenced that testes of subordinate and inactive breeding males are of smaller size compared to dominant breeding bulls. The echogenicity of the parenchyma also changes, with the active gonad being less echoic due to higher blood circulation compared to the inactive one.

Figure 31: Ultrasound image of the right testicle (Courtesy of Dr. Thomas Hildebrandt, Department of Reproduction Management – IZW, Germany).



2.2.2 Male Reproductive Physiology

Puberty in male elephants occurs between 9-15 years of age, reaching maturity at the age of 17 (Evans & Harris., 2008). However, they are unlikely to mate until their late teens or twenties. Mating is a relatively brief act, requiring the female to stand while the male mounts and copulates. The male may use his trunk to position or hold the female, but her cooperation appears necessary for successful intromission. Mounting is practiced not just by reproductively active males, but also by younger elephants, perhaps as a sign of dominance or as a mean of learning (Schulte, 2006).

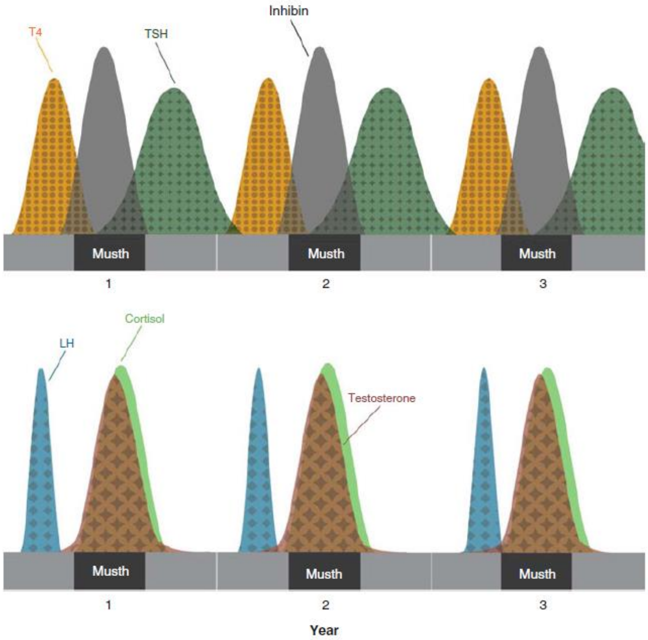
Musth is a complex hormonal phenomenon that occurs in general annually in adult Asian and African bulls and can last between 1 – 5 months (Hildebrandt et al., 2006), although its occurrence can be random and bulls may exhibit multiple episodes of musth per year (Brown et al., 2007). It is not considered a seasonal routine event and moreover it is associated with increased aggression, breeding, and changes in androgen secretion. Testosterone levels often increase up to 20 times of the normal value (Rasmussen & Krishnamurthy, 2000). On a study performed by Brown et al. (2007), testosterone was generally <10ng/mL in bulls not in musth (e.g. castrated) or during inter-musth periods, and musth would be detected only when concentrations would rise above 50 ng/mL and often exceeding 100ng/mL. Findings also show that there is a marked increase in the testosterone levels around 20 years of age, and concentrations are higher in older dominant males. Cortisol secretion presents a positive correlation with testosterone in males in musth (Figure

33). However, TSH and thyroid hormones (T3, T4) were negatively correlated with testosterone, for the bulls presenting yearly musth cycles (Figure 33). On the same study, thyroid hormones presented seasonal effect only in the African elephant, showing lower concentrations during summer months (Brown et al., 2007).

Since musth also occurs in castrated males, it may be associated with other unknown factors. During musth, a complex array of chemical compounds is released from the breath, urine, and temporal glands, capable of inducing musth status in other male elephants. Males in musth are considered dangerous and unpredictable, and therefore people working directly with elephants should be able to recognize this phenomenon. The temporal gland secretion, visible as dark liquid tracts on the sides of the face (Figure 32) is considered the main primary sign, being also important the perineal swelling, constant dribbling of urine, and a fetid odour. Males in musth may stance with their head being held more erect and the ears extended. Characteristic vocalizations called “musth rumbles” may be heard. During this period, males often stop eating, losing a large amount of weight. In this condition, they also will not breed, a way in which musth differs from heat. Young sexually immature males sometimes secrete a sweet-smelling liquid from their temporal glands - referred to as a “honey musth” or “moda musth.” Studies on these fluids indicate that they contain a complex mixture of pheromones (Rasmussen & Krishnamurthy, 2000). However, it seems that despite being in the ranging age and physical maturity, not all bulls present obvious signs of musth; not showing temporal gland secretion, urine dribbling, increase on testosterone level, or aggressive behavior. Therefore, there is no one profile that fits all bulls (Brown et al., 2007).

Figure 32: Temporal gland secretion in African bull in musth (Source: http://www.bbc.com/earth/story/20160614-the-people-who-ate-elephant-heads)

Figure 33: Scheme of the hormonal changes in testosterone, LH, cortisol, inhibin, TSH and T4 during musth in elephant bulls (Brown, 2014).



2.2.3 Musth management

Due to an increase in aggressive behaviour of captive males during the musth period, it is imperative to create safety measures to protect the animal and involved staff.

There are different approaches to deal with this situation. Schmitt (2006) reminds that musth is a natural phenomenon in healthy adult bulls, and therefore decisions should be made with caution.

A possible alternative for controlling or preventing musth is by chemical control with leuprolide acetate, a gonadotrophin releasing hormone (GnRH) agonist, however permanent reproductive effects may occur. Injections should be given during early or pre-musth. Initially, a significant increase in testosterone levels is achieved which will subsequently decline over 10-14 days, due to down regulation (Oliveira et at., 2004).

2.2.4 Pathology of the male reproductive system

Previously, poor reproductive performance of captive elephants was normally attributed to female dysfunction, but studies suggest that fertility problems of bulls may be of equal or greater importance; the majority of the captive male individuals are probably not capable of producing offspring. Besides poor libido, healthy adult bulls are not producing good-quality sperm, excluding the option of using them for artificial insemination (Hildebrandt et al., 2000b). Due to the gender ratio present in captivity, male infertility has a negative impact approximately ten times higher than female reproductive disorders (Hildebrandt, 2000c).

Accessory sex glands can be involved during urogenital infections such as by Escherichia coli, or in a reported case of pathological changes caused by a chronic prostatovesiculitis – here, the infected prostate was enlarged, with a less echoic parenchyma and the concurrently infected seminal vesicles presented a thicker wall (> 10 mm) and contained a cloudy secretion. In this clinical case, microbiological analysis of the ejaculate revealed urogenital infection and samples were of chocolate colour, deviating from the normal white to amber appearance (Hildebrandt et al., 2000b).

Hildebrandt et al. (2000b) shows that some adult bulls present with testicular pathology of unknown origin, including poor semen quality and small testes (<10 cm), with echogenic parenchyma on ultrasound examination. Testicular cysts may also be observed.

Possible causes for the testicular degeneration are:

- Chronic infection;
- Long-term treatments for diseases (such as tuberculosis);
- Inadequate application of anti-musth drugs.

The return of these bulls to an active breeding status is questionable due to frequently reported massive changes in testicular morphology, registered during post-mortem examination.

Urethral alterations, such as calcification of the mucosa, seem to be rare. Urethrostomy performed for surgical removal of the stone resulted in a permanent urethral fistula similar to that seen in females after episiotomy for dystocia treatment. No ultrasonographic evidence of urinary bladder infection is reported in bulls (Hildebrandt et al., 2000b).

Asymmetric reproductive ageing and premature senescence do not seem to affect males, but unsuitable social environments may impact their reproductive performance and fertility. Therefore, social stress or intense physical activity seems to be the most probable cause of the negative effects of long-term captivity commonly found on male fertility (Hildebrandt et al., 2000c; Hermes et al., 2004).

2.2.5 Chemical communication in elephants

Elephants seem to communicate their fertility state and cycling stage through chemical signals that differ according to the species (Brown, 2014): the female Asian elephant produces a urinary signal, (*Z*)-7-dodecenyl acetate (*Z* 7-12:Ac), which increases before the second LH surge, peaking just before ovulation. This pheromone becomes detectable in the urine after the luteal serum progesterone concentration decreases to baseline levels, and its concentrations increase during the follicular period, with no association with the second LH peak. This female pre-ovulatory urinary pheromone indicates readiness to mate and seems to stimulate male breeding behaviour (Rasmussen, 2001).

In the African elephant, Goodwin (2006) states that *Z* 7-12:Ac is not produced, and instead the female excretes frontalin, exo-brevicomin, endo-brevicomin, (*E,E*)-α-farnesene and (*E*)-β-farnesene in the urine, before and during oestrus.

When a female is found in oestrous, bulls can switch urine dribbling on within hours, and stop it within minutes at the arrival of a higher ranking musth bull. The association between high androgens, sexual activity and musth signals manifested by the bull suggest that oestrous females may act as triggers; prolonging the periods of sexual activity and musth during seasons with many oestrous females, or contrarily, reducing the periods during suboptimal seasons (Rasmussen et al., 2008).

Chapter 3 Assisted Reproduction Technology

3.1 Reproductive evaluation of the elephant bull

For accessing the breeding potential of captive bulls, a semen collection is made by a variety of methods. Typically, it is achieved by manual rectal massage, with or without standing sedation, but artificial vagina (AV) or electroejaculation may also be used (Kiso et al., 2013).

Ultrasound examinations should be combined with semen collection and conducted yearly in order to assess the reproductive status and to determine if changes in management strategies should be made (Hildebrandt et al., 2000b). Ultrasound is also useful to evaluate the status of the *ampullae* glands before and after ejaculation and can be used to verify if they are completely emptied. When attempting to collect semen from bulls of unknown status, it helps to evaluate the ejaculatory response (Schmitt & Hildebrandt, 1998). This approach facilitates the identification of bulls that could serve as semen donors for future artificial insemination (Hildebrandt et al., 2000b).

Ejaculation occurs in multiple aliquots that may vary considerably in quality. The semen quality, motility, and concentration collected from a single bull by manual rectal massage may also vary on a day-to-day basis, and its unpredictability adds another challenge to AI (Kiso et al., 2011).

Since handling of bulls is often too dangerous for routine blood collection, especially during musth periods, non-invasive monitoring of testicular function by urinary or faecal androgen metabolite analysis provides a safe route of obtaining this information. In consonance with the females, long-term endocrine monitoring is important also in males, especially as more bulls are being diagnosed with reproductive problems (Brown, 2000).

3.2 Semen Collection

Semen samples can be collected and preserved from both wild and captive populations to maintain a genome resource bank and to boost reproduction in these endangered mammals (Hermes et al., 2009). The different methods used for semen collection in elephants are presented below.

Artificial vagina

The use of an artificial vagina in elephants requires a trained behaviour. The bull is restrained with all four feet on the ground. A partially erected penis is inserted into the AV and the ejaculate is collected. The sample is highly concentrated, similar to that found during rectal massage. This results in an average volume of 30 mL of ejaculate in 60 - 80% of the bulls (Schmitt, 2006).

Electroejaculation

Semen collection through electroejaculation requires a specially designed, hand-held electrical probe (Figure 34), which with assistance of US guidance, is used to induce electric stimuli to achieve ejaculation. After each set of stimulations, a manual massage of the pelvic and penile urethra is executed (Figure 35). Semen is collected to a thermal insulated 50 mL vial, located at the end of a collection bag that is placed over the penis. The collection tubes are changed regularly as a preventive measure to minimize urinary contamination (Hermes et al., 2013).

Figure 34: Electroejaculation probes used in large animals. On the left back we can see the one used for semen collection in elephants; the probe in the front is normally used in humans, for comparison. (Image source: http://michaelmanyak.com/wp-content/uploads/2014/05/5probes.jpg).

Figure 35: Exteriorized penis during electroejaculation with condom for semen collection (Courtesy of Dr Thomas Hildebrandt, IZW, Berlin).

Manual stimulation

The manual stimulation through rectal access technique is explained in detail by Schmitt & Hildebrandt (1998). In Chapter 7 the procedure is also stated, as semen collections were performed in two bulls during the reproductive examinations.

3.3 Semen evaluation and laboratory processing of the semen

In a study performed by Schmitt & Hildebrandt (1998) in a total of 250 collections over a 10-year period, 80% of the semen collection attempts reported good quality and adequate quantities of the ejaculate. Good quality semen presents white colour, and usually the first fraction collected is the most concentrated one (Table 3). The third fractions were often yellowish and presented lower concentrations of sperm cells and several urinary salts (on microscopic examination), and were therefore discarded. The samples were considered not to be influenced by season or the presence or absence of musth (determined by drainage from the temporal glands).

The protocol for processing of the semen samples is presented in Figure 38.

Table 3: Average semen characteristics, analysed from samples collected by rectal massage; mean volume, concentration and pH for the first and second ejaculatory responses (Schmitt & Hildebrandt, 1998).

Fraction	Volume (mL)	Concentration ($x10^9$/mL)	pH
1st	10.5	2.05 ± 0.17	7.05 ± 0.07
2nd	17.0	1.34 ± 0.19	7.04 ± 0.13
Total	27.5	1.61 ± 4.4	

Elephant sperm cells are smaller than those found in other domestic animals (Table 4) and contain dense bodies not seen in other species. Sperm membranes of African elephants are composed of more unsaturated, long-chained fatty acids compared to those found in Asians elephants (Schmitt, 2006). Compared to domestic ruminants, the acrosomal ridge is difficult to visualize using light microscopy, but it may be stained with various stains for acrosomal integrity and morphologic changes (Figure 36 and 37). The equatorial segment extends distally, on the ventral side of the head. In the neck region, dense material is located near the top of the mitochondria; these function of these dense spherical masses is unknown (Wattananit, 2014).

Table 4: African elephant semen characteristics (Hähnel, 2007)

Head width	4,1 ± 0,3 µm
Head length	6,9 ± 0,6 µm
Tail length	54,1 ± 1,7 µm
Relationship tail length to head length	7,8

Figure 36: Asian elephant sperm morphology (using a magnification of 1000x) A) Normal; B) Proximal cytoplasmic droplet; C) Abnormal mid-piece; D) Tightly coiled tail; E) Bent tail with cytoplasmic droplet; F) Spermac staining (solid arrow: Spermac positive; dotted arrow: Spermac negative) (Kiso et al., 2013).

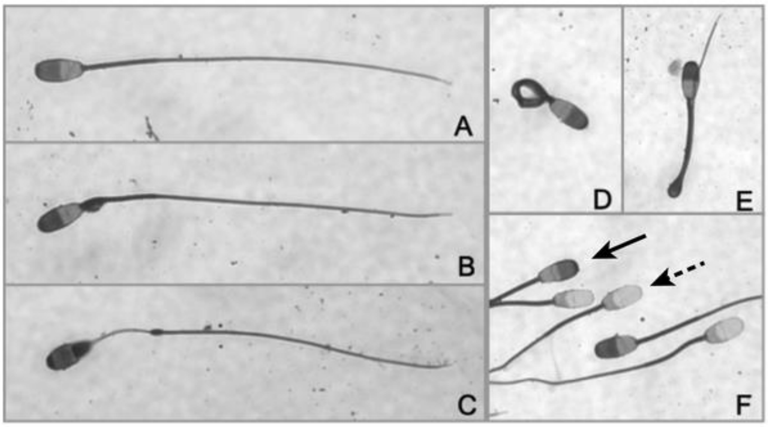


Figure 37: Spermac staining of elephant spermatozoa. Observations should be made using a light microscope (1000x) using oil immersion (Image Source: https://elephantconservation.org/programs/captive-programs). Stain explanation: Acrosome – Dark green, Nucleus – Red, Equatorial region – Pale green, Midpiece and tail – Green.

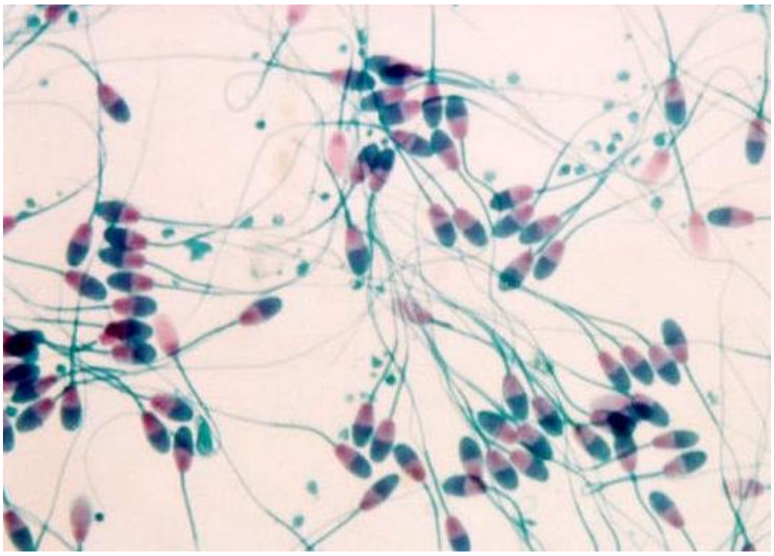
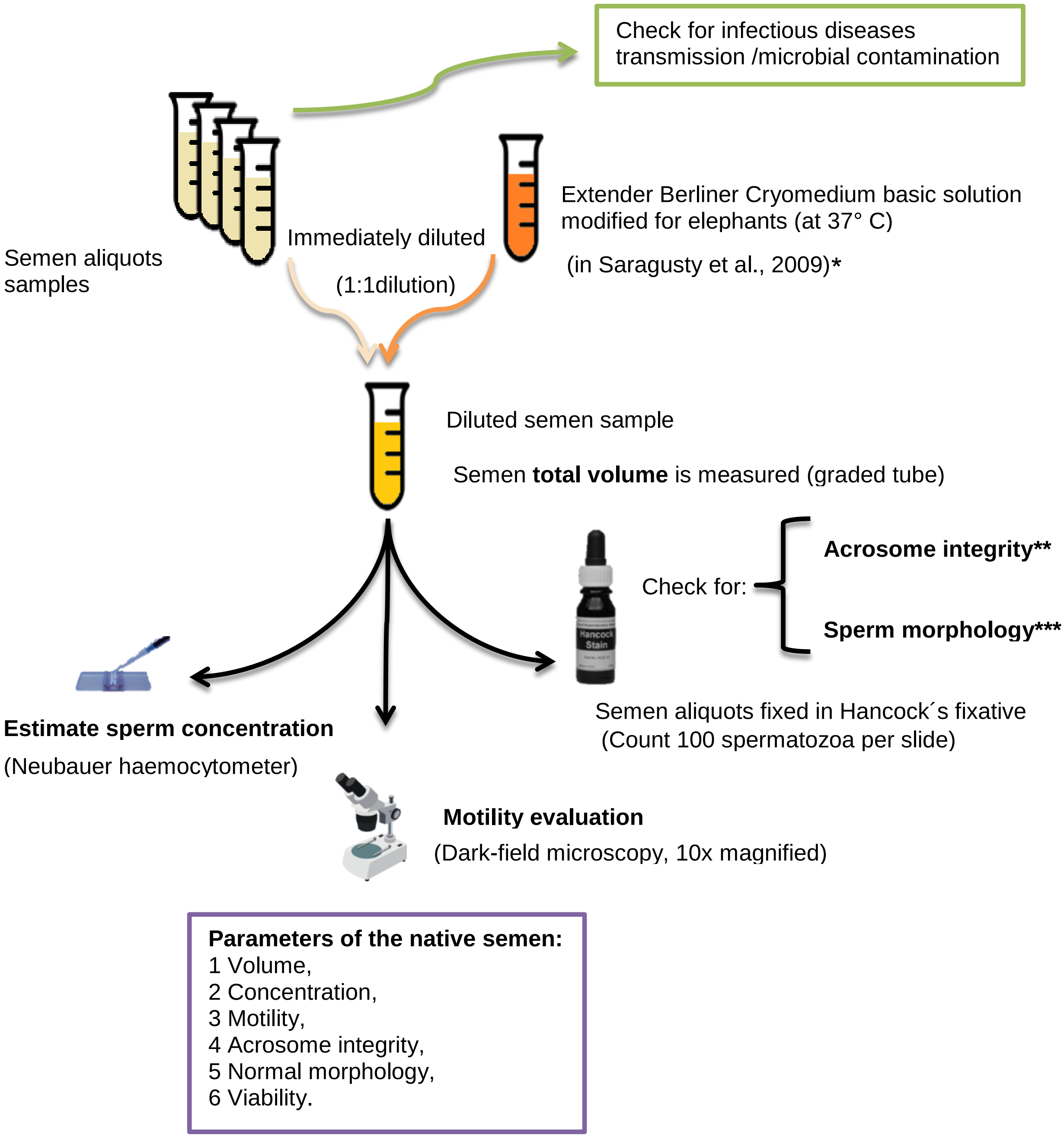


Figure 38: Scheme of semen processing after collection (Original, information source Hermes et al., 2013).



* Technique initially developed for Asian elephant (see more information in Saragusty et al., 2009).
** Tests for intact vs modified or reacted acrosome (including complete detachment).
*** Includes search for abnormalities.

Semen samples should also be analysed for viability; using coloration with nigrosin – eosin the dead sperm cells can be distinguished, which allow the stain to penetrate the damaged membrane, while live spermatozoa with an intact membrane remain unstained (J. Saragusty, personal communication, 2015).

3.4 Preservation of spermatozoa

Fresh chilled elephant semen is commonly used in AI programs in order to aid elephant breeding and genetic management in captivity. Transportation of chilled preserved semen should be made at a stable temperature of 4°C. Here, the use of an Equitainer® (Hamilton Research, Inc.) presents good results, maintaining semen fertility for 72 hours (T. Hildebrandt, personal communication, 2015; http://www.equitainer.com/). However, with the use of this short term preservation, semen has to be collected close to ovulation time.

Sperm viability may deteriorate rapidly within 12 hours after collection. The use of different semen extenders and novel freezing protocols to improve and extend elephant semen quality is an area of active research (Kiso et al., 2011).

Cryopreservation is more successful in African elephants, since semen from these species tolerates more basic extenders, while Asian elephant semen seems to be more susceptible to spontaneous acrosome degeneration, needing a more complex extender (Schmitt, 2006).

Hermes et al. (2013) collected semen by electroejaculation from wild *L.africana* bulls and created a new protocol, using the directional freezing technique. Specifically, the cryopreservation method includes centrifugation, to remove the seminal fluids, and then dilution in BC extender with hen egg yolk and 7% glycerol. However, some uncertainty remains as samples were collected during different seasons and from different bulls, and the results of this study indicate that the collection at the end of the rainy season produces better quality of sperm compared to the dry season. Sperm cryopreserved using this method already lead to a pregnancy of an artificially inseminated captive female elephant (Hermes et al., 2013).

3.5 Artificial Insemination

Artificial Insemination is especially important when female elephants have no access to males or if the animal is not compatible with natural mating. The success rate in elephants is around 30-40% for each attempt which is lower than the 50-60% found for natural breeding (Schmitt, 2006). Since 1998, AI has been used in captive elephants, and more than 40 pregnancies have been achieved using this procedure (T. Hildebrandt, personal communication, 2014).

3.5.1 Insemination timing

Monitoring the oestrous cycle in a captive environment, allows to correctly time both natural mating and AI. Daily sampling should be done 30 to 25 days prior to the expected ovulation day in order to detect the first LH surge. Fourteen days after the detection of the first peak, new samplings should be made to detect the second LH peak, when ovulation will occur (T. Hildebrandt, personal communication, 2014).

Lueders et al. (2010) report that subordinate bulls attempt to mate around the first LH surge, while more experienced breeding bulls mate 2–3 days prior to the day of the second LH peak and mating behaviour stops as soon as ovulation has occurred. This reveals important implications for the timing of AI, suggesting that this technique has higher likelihood of being unsuccessful once the follicle has already ruptured.

Typically, the female is inseminated twice: the day of and the day after the second LH peak. For natural breeding male and female elephants are housed together during these days (Hildebrandt et al., 2006).

US is an important tool in AI, allowing the inseminators to follow the follicular development, predict the ovulation time, confirm that ovulation has occurred, and monitor the site of semen deposition during the procedure (Schmitt, 2006).

3.5.2. AI procedure

A female elephant is considered a good candidate for artificial insemination if she has the following characteristics:

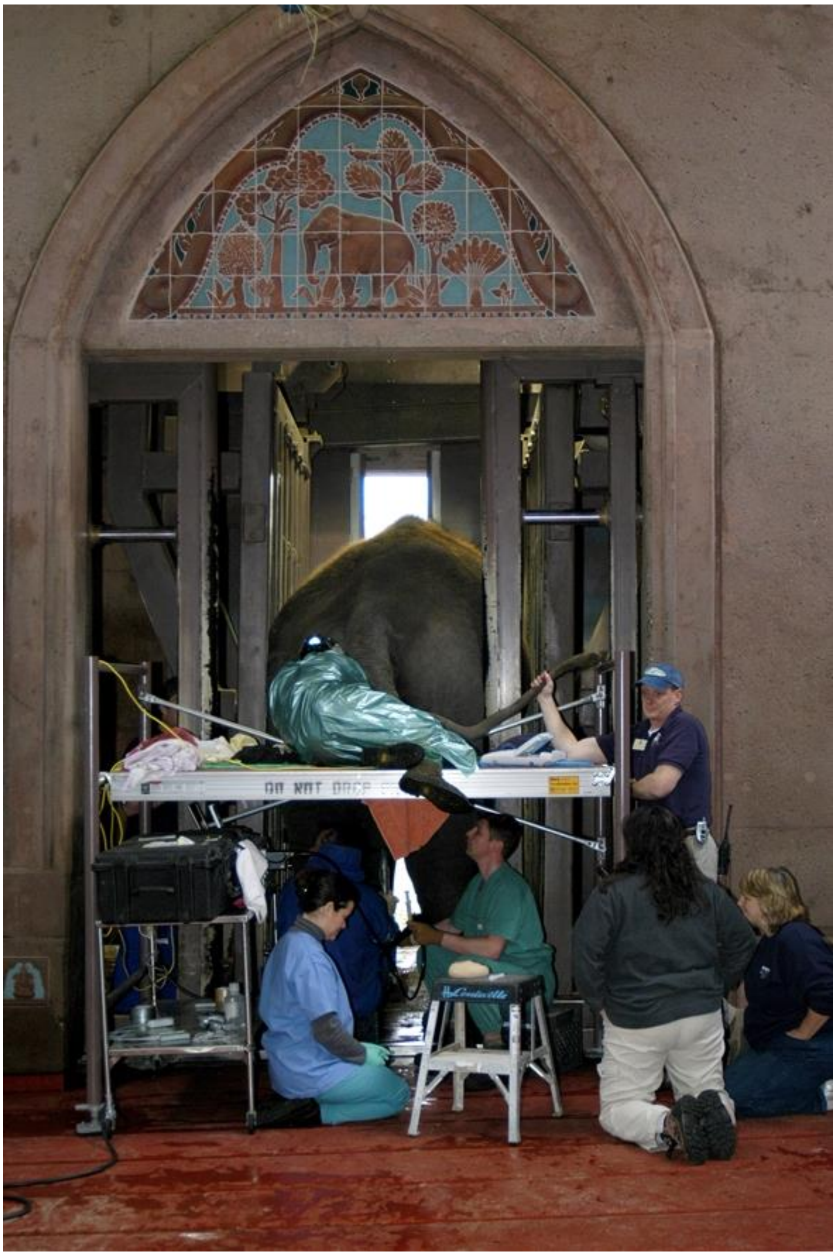
1. At reproductive age known to present maternal behaviour;
2. Good body and health condition;
3. Normal reproductive cycle;
4. Tolerated daily and weekly blood sampling;
5. Responds well to training.

(https://nationalzoo.si.edu/SCBI/ReproductiveScience/ElephantBreedRepro/AfricanElephantSurveyStudy/default.cfm, acceded on 15 August 2016).

A compliant cow is restrained and a custom-made balloon catheter, similar to a large tracheal tube (140 cm length), is lubricated with non-spermicidal sterile gel, and inserted into the vertical part of the vestibule. Through this catheter, a flexible video endoscope (2.5 m) is inserted. Then a small insemination catheter can be placed in the working channel of the endoscope (3 mm in diameter and 300 cm in length). Both endoscopic and ultrasonographic visualisation are used to guide the semen catheter into the hymen opening, and the semen is deposited in the distal vagina, or in parous cow through the cervix directly into the uterus (Brown et al., 2004b; Schmitt, 2006) (Figure 39).

A surgical approach can be used in cows with polyps or in cows that do not tolerate the above described technique. The surgical insemination is considered technically easier, performed by making a 1 cm long incision below the anus, to access the urogenital canal. With the help of an equine sterile vaginal speculum, semen can be deposited. Tree to four sutures are used to close the surgical site, and after care should be maintained for 4-6 weeks until complete healing is accomplished and sutures can be removed (Schmitt, 2006).

Figure 39: Artificial insemination in Woodland Park Zoo. Note that on the elevated structure the veterinary is controlling the process with ultrasonography to check if the insertion of the small insemination catheter and the deposition of semen is in the correct place. On the ground the other operator has access to the vestibule and manipulates the endoscope (Credit: Ryan Hawk; accessible at http://voices.nationalgeographic.com/2011/08/05/elephant-artificial-insemination/).



3.6 In vitro fertilisation (IVF), Intra-cytoplasmatic sperm injection (ICSI) and Embryo transfer

For infertile female elephants with a long history of reproductive lesions, oocyte collection and in vitro assisted reproductive technologies represent important tools to preserve their genetic information and to contribute to the genetic diversity of a population. Although the technique is nowadays widely used in domestic species, and follicle aspiration is achieved by laparoscopy or by transvaginal US-guidance, Hermes et al. (2007) point out that these techniques are non-functional in megavertebrates. The anatomical dimensions remain a major challenge for the development of IVF, ICSI as well as embryo transfer in elephants. The same author states that although laparoscopic access has been achieved in these species, the skin healing abilities, the high abdominal pressure and the risk of post-surgical peritonitis, exclude a surgical approach for repeated oocyte collection in mega-vertebrates. Also transvaginal endoscopic oocyte collection, is still considered a non-practical approach due to insufficient insufflation of the large abdominal cavity, the difficulty of visualisation of the ovaries, the resorption of CO_2 into tissues and subsequent intestinal and abdominal wall emphysema (Hermes et al., 2007).

3.7 Sperm sex sorting

Hermes et al (2009) have recently demonstrated the possibility to sort Asian elephant sperm into X- and Y-chromosome bearing sperm populations with the aid of flow cytometry. However, the sorting process is slow and would take many hours to produce a sample large enough for artificial insemination. Combining this technique with sperm freezing and artificial insemination may help not only to counter skewed sex ratio trends, but also through insemination with X–chromosome-bearing spermatozoa, increase the proportion of females in the population and therefore the production of offspring (Hermes et al., 2009).

3.8 Hormone cycle induction

A study performed in Asian elephants induced ovulation by administering an GnRH agonist (GnRH-Ag) (with Buserelin-Acetat, 80µg intravenously) during the late anovulatory follicular phase; 90% of the females responded with an immediate LH surge which followed 15–22 days later by an ovLH surge or a post-ovulatory increase in progestogens. Administration of this drug in the ovulatory follicular phase and the luteal phase created an immediate increase in LH, but no subsequent ovLH surges were registered (Thitaram et al., 2009). Therefore, the GnRH-Ag is able to induce a predictable ovLH surge, although with LH concentrations lower than normal LH surges. The effects of this agonist on the pituitary and gonadal function in elephants are still unknown (Brown, 2000). This protocol shows potential for reducing the effort, time and number of samples needed to identify the anLH surge in order to properly time natural and assisted breeding, thereby increasing the captive breeding management of Asian elephants (Thitaram et al., 2009).

Another option to ensure ovulation at the time of the AI is the use of human chorionic gonadotropin (hCG) (5000 IU); injecting it at the time of the last AI procedure is reported to induce rupture of the dominant follicle. However, the follicle needs to reach a certain size or stage of maturation in order to be receptive for ovulation induction. Administration of hCG has also led to follicular stimulation in one case, with ovaries presenting more than 15 follicles each (Hildebrandt et al., 2011).

3.9 Hormonal contraception

In older female Asian elephants, suppression of ovarian function may be recommended due to their predisposition to develop uterine leiomyoma, especially when in non-reproductive status (Hermes et al., 2004).

In free ranging elephants the most practical and applied contraceptive agent is the porcine Zona Pellucida (pZP) vaccine: it causes an immune response targeting mature oocytes. With the use of this immunogenic preparation the intercalving period increases, but there are concerns on its reversibility on whether the ovarian tissue is also affected. The second contraceptive method used both in the wild and in captivity, involves oestrogen implants combined with melengestrol acetate implants (MGA), leading to an effective inhibition of ovulation, prolonging the inter-calving period, and a complete inhibition of luteal progestogens (when serum levels reaches 20-30 pg MGA/mL). Results indicate that this human "mini-pill" strategy can be used in elephants. For these hormonal contraceptives no adverse effects on the uterus or ovaries have been found during ultrasound examination (Hermes et al., 2007; Hildebrandt et al., 2006)

The GnRH vaccine is an inoculant designed to induce an immune response against the endogenous hormone GnRH, and it is used in a variety of animal species to control fertility. The antibodies will neutralize and prevent GnRH from taking its action, since it is unable to bind to the pituitary receptors. Subsequently, the release of LH or FSH is supressed, the stimulation of testicles or ovaries will cease, and the gonads become inactive. GnRH vaccine is recommended as a non-surgical method to control testosterone levels and musth in elephant bulls or to prevent uterine pathologies in females. However, very little is known about dosages, administration intervals, and long-term and side effects in elephants. Long term studies are needed for male and female elephants to check for possible pathological effects. After treatment, several species are reported with severe testicular degeneration, which it is also observed in elephants, comprising a size reduction by up to 60%, amorphous shape, dilated blood vessels and fibrosis, as well as penis atrophy. Few data are available on the effects of the GnRH vaccine on female elephants, and studies are currently conducted to understand the impact of this hormonal vaccine in preventing, receding or stopping leiomyoma growth (Lueders & Oerke, 2016).

Chapter 4 Pregnancy and Parturition

4. 1 Introduction to pregnancy

In free-ranging elephants first conception occurs at around 10-12 years of age. In captivity, females achieve puberty at an early age and present elevated growth rates, possibly due to nutritional differences (Schmitt, 2006). Captive elephants have been reported to be pregnant as early as 3.5 years of age for the Asian elephants, and 7 years for the African species (Hildebrandt et al., 2011). Gestation in elephants lasts around 640 – 660 days and single births are common, with a twinning rate of only 1 – 2 % of births (Schmitt, 2006). Elephants have an inter-calving period approximately 3 – 7 years (Hildebrandt et al., 2006).

Ultrasonography and endocrine assays play a key role also in the evaluation of the gestating cow, e.g. determination of the number of foetuses and the impending moment of parturition (Schaftenaar & Hildebrandt, 2005) and allow for achieving a better understanding of the physiology of pregnancy in elephants (Brown, 2014).

4.2 Physiology of gestation

Progestogens

During gestation, the progestogen secretory pattern differs between elephant species. In African elephants, serum progestogen concentrations rise from the time of ovulation and drop pronouncedly at around 5 – 6 weeks after conception (at the time of embryo implantation). Another peak is achieved around the 5th month of gestation, remaining relatively constant for the next 5 – 6 months, and declining again sharply to a lower plateau, until the sudden final drop, 1 – 2 days before birth (Allen et al., 2005; Meyer et al., 2004). Therefore, the African elephant female presents a higher concentration of progestogens during the first half of pregnancy, while Asian elephants show a slower increase, but a more parabolic secretory curve (Figure 40) (Meyer et al., 2004).

Elephants and horses share some gestational traits, one of which is the presence of multiple large CL in the maternal ovaries (Stansfield & Allen, 2012). Nevertheless, there are notable differences: in the elephant, the acCLs are produced throughout the follicular phase of preceding cycles (Lueders et al. 2010; 2011), and there are no additional CLs produced during gestation (Lueders et al. 2012). However, Schmitt (2006) states that during mid and late gestation, the acCL number increases.

Allen et al. (2002) investigated, the endocrinological aspects of placental and fetal tissues in 59 African pregnant elephants, and discovered that no progestogens or oestrogens were secreted by the placenta. Because of this endocrine inactivity in the zonary elephant placenta, he suggests that to be able to maintain the gestation there should be a maternal luteal source of progestogens.

Lueders et al., (2010) propose that elephants have evolved a strategy that differs from all other mammals, and that by using the two LH peaks for CL recruitment, elephants can ensure to have sufficient luteal capacity to maintain their long-lasting pregnancy. In 2012 the same author performed a study during pregnancy and suggests that unlike in other species, where a free embryo sends signals for maternal recognition, in elephants it seems that only with the attachment of the hatched, healthy and viable blastocyst a signal is transmitted, stopping further CL regression and inducing additional CL-growth. Therefore, the amount of luteal structures, which enlarges only after implantation, allows the dam to only invest in a viable embryo (Lueders et al., 2012).

Asian elephant dams carrying male calves present higher progestogen concentrations when compared to those gestating female calves. This foetal gender effect was not found in African elephants (Meyer et al., 2004). However, after anatomical and histological studies performed in ovaries and testes of foetal African elephants, it is proposed that progestogens are secreted by the enlarged gonads of the elephant foetus, which may function both to assist the maternal ovaries in supporting the pregnancy, and to induce torpidity and intrauterine immobility of the growing foetus (Allen et al., 2005; Stansfield & Allen, 2012).

Relaxin

The concentration of relaxin rises constantly until 10 months of gestation, declining gradually from this point, and finally peaking a few weeks before birth occurs. African and Asian species present the same secretory pattern of relaxin (Meyer et al., 2004) (Figure 40).

Prolactin

Most literature states that PRL levels increase markedly from around 6months of gestation and remain high until parturition (Figure 40). Contradictorily, Yamamoto et al. (2012) studies present a clear biphasic PRL pattern during pregnancy in African and Asian elephants; the circulating levels start to increase at 4 – 6 months of gestation, reaching a first peak at 11 - 14 months, and a second peak at around 18 – 20 months, followed by an abrupt decline just after parturition. This author states that this difference found in reported PRL secretory patterns, is likely due to a higher sampling frequency, and due to the higher sensitivity of the RIA when compared with the previously used hormone assays.

Immunoreactive PRL (ir-PRL) was found in the placenta, more precisely in the trophoblast, of African and Asian elephants. This suggests that in elephants the placenta is one of the major sources of PRL, at least during the late gestation period, whereas the source of the first PRL peak is still unknown. Conversely, in case of abortion of one African elephant, the second PRL peak was not registered (Yamamoto et al., 2012). It was also proposed that

PRL has the capacity to stimulate the rapid growth and secretory function of the previously “dormant” accessory CL, ensuring the persistence of the CL throughout gestation, (Yamamoto et al., 2011; 2012). Nevertheless, is unlikely to be involved in the initial formation of the luteal structures (Allen et al., 2006).

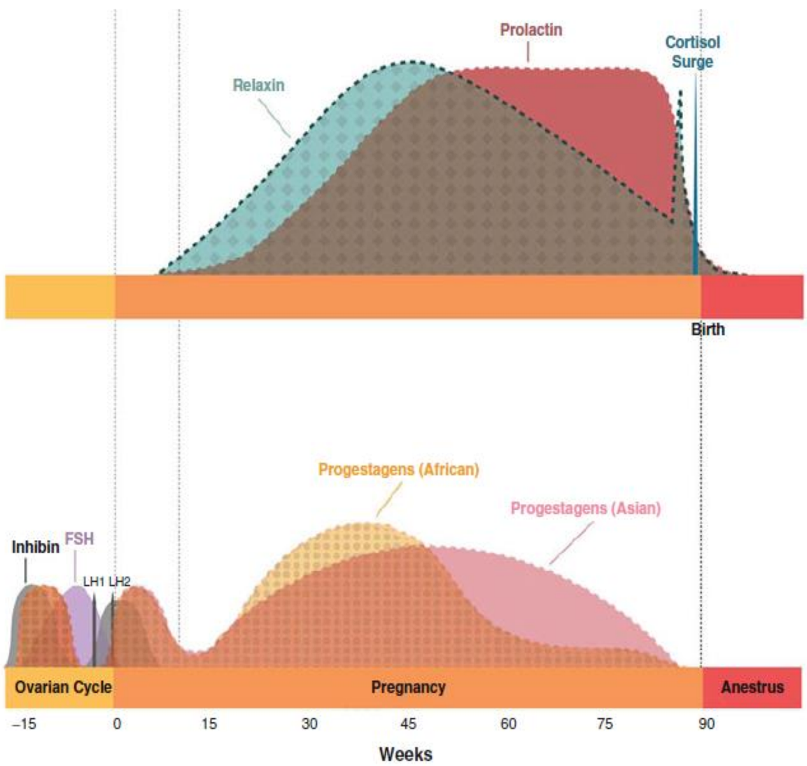
Cortisol

No changes are observed in cortisol levels observed throughout the entire gestation, with a steep increase only shortly before parturition: 5 – 10 days for the African elephant, and 30-40 days for the Asian elephant (Yamamoto et al., 2010) (Figure 40). Therefore, cortisol plays a rule in the initiation of parturition (Meyer et al., 2004).

Inhibin

Serum levels of inhibin are low throughout the gestation period, when compared with its concentration at the peak during oestrous (Yamamoto et al., 2012). Inhibin reaches high levels only between 7 – 10 weeks of gestation, at the time of embryo implantation (Drews et al., 2008) (Figure 40).

Figure 40: Scheme of secretion of progestagens, LH, FSH, prolactin, relaxin, cortisol and inhibin during the gestation, and during a prior oestrous cycle in the elephant (Brown, 2014).



4.3 Pregnancy diagnosis

A weekly test should be performed to attain a pregnancy diagnose, and throughout the entire pregnancy. Pregnancy can be diagnosed by:

1. Progestogen measurement; Diagnosis and monitoring of pregnancy are easily done by longitudinal analysis of 5α-reduced pregnanes in the bloodstream or the relevant metabolites in urine or faeces (Brown, 2000; Hildebrandt et al., 2006). In the first 6 weeks of pregnancy, the progestogen level does not differ from a normal luteal phase of an oestrous cycle and even a temporary decrease to nearly baseline value occurs around 8 weeks post conception. However, the level of this hormone exceeds normal cycling values at around 10 – 12 weeks after initiation of pregnancy, reaching even 2 – 3 times the normal values during the first half of gestation (Brown, 2000). Progestogen levels stay high until 3 – 5 days before parturition (rare cases longer) (Hildebrandt et al., 2006). Therefore, shortly before parturition progesterone monitoring should be performed at increased frequency. Quick results are needed to predict the impending birth and to give time for adequate preparations (T. Hildebrandt, personal communication, 2014). Measurements of progesterone levels at the end of pregnancy should preferably use blood, rather than in urine, as more laboratories are available for serum testing (Schaftenaar & Hildebrandt, 2005).
2. Prolactin measurement; approximately 6 months post-breeding prolactin values can rise 200 - 600 times above normal cycling values (Brown, 2004).
3. Ultrasound examinations; in case of pregnancy, ca. 50 days after ovulation, the embryonic vesicle can be visualized trans-rectally within the uterine horn (Drews et al, 2008). After 250 days of gestation, trans-abdominal visualization of the foetus is possible (Lueders & Hildebrandt, 2012).

One of the first visually observable indications of pregnancy is the mammary development. Breasts are slightly swollen after 6 months of gestation; the increase in glandular firmness and size is detectable by palpation. With the progression of gestation, the mammary glands become more enlarged (Schmitt, 2006).

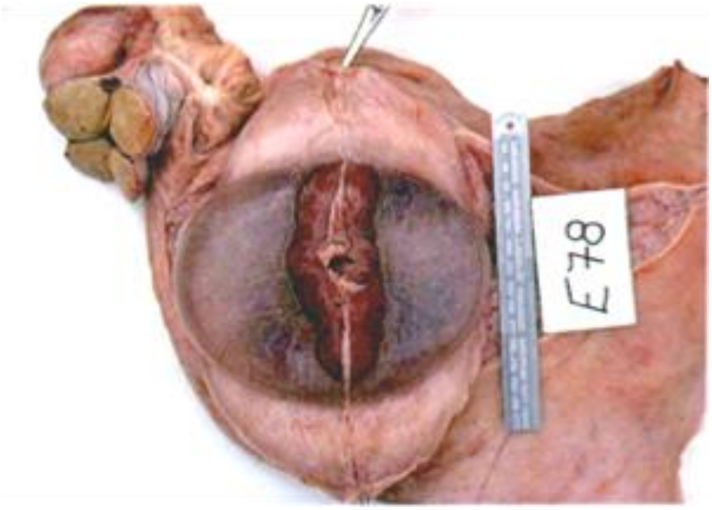
If progestogen levels are appropriately elevated and the elephant is otherwise healthy, the inability to find the foetus by using ultrasonography is likely not a concern, since the foetus may be extremely difficult to visualize. In such cases another examination may be scheduled for a later date (Hildebrandt et al., 2006).

Between 30 and 60 weeks of gestation, foetal gender may be determined by measuring testosterone levels in the cow’s blood of Asian elephants (this is not yet reliable for African elephants). After 8-12 months of pregnancy, sex determination is also possible with the use of ultrasound (Hildebrandt et al., 2006).

4.4 Placentation

The elephant placenta develops exclusively by stromal growth of the surface of the maternal endometrium. The final dimension of overgrowth is limited to a small band of maternal and foetal tissues. Elephants possess a zonary and endotheliochorial placenta (Hildebrandt et al., 2006), thus calves are born immune competent, as they receive antibodies received transplacentally (Nofs et al., 2013). Although a vast amount of growth and modifications occur during pregnancy, the width of the hilus does not significantly increase. To support the increasing needs of the growing placenta and foetus, more and bigger blood vessels develop in the endometrium (Figure 41). This concentrated vascular plexus may bleed profusely if sudden rupture occurs (Allen et al., 2003). At term, the elephant placenta is large and usually weighs between 15 – 24 Kg (including the free membranes) (Allen et al., 2003) The chorioallantoic membranes are covered with “verrucae” (Allen, 2006).

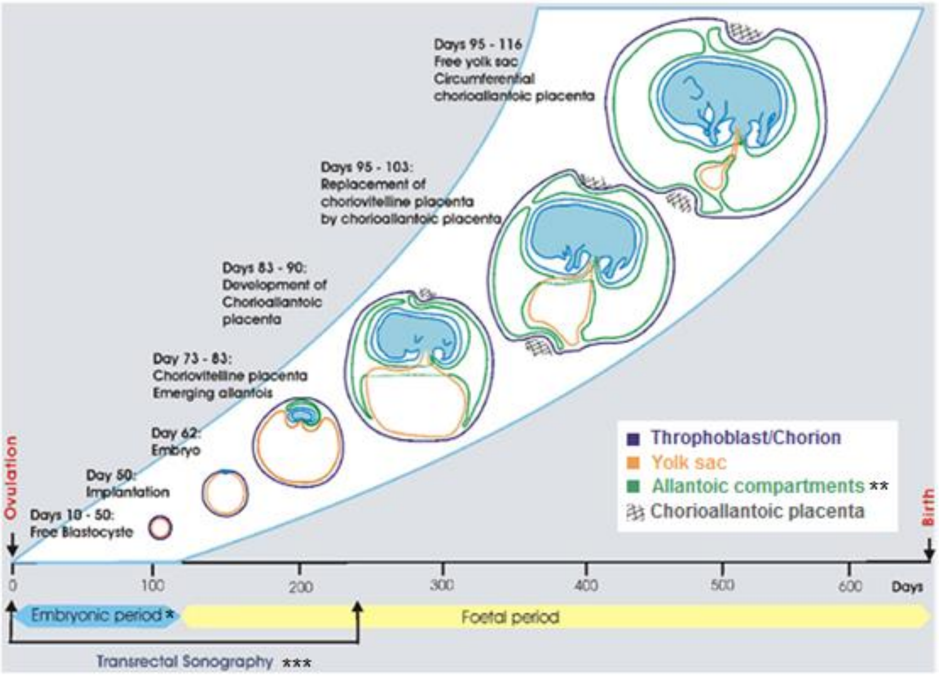
Figure 41: Elephant conceptus at 11 month of gestation; zonary placenta, passing in the equatorial region and presenting a narrow hilus, which attaches the embryo to the endometrium (Allen et al., 2002).



4.5 Embryonic development

Hildebrandt et al. (2007) studied the early stages of pregnancy in Asian and African elephants by using transrectal ultrasound and through measurements of foetal crown–rump lengths. Thereby, they provide the first accurate growth curves, which differ significantly from the previous theoretical estimates based on the cube root of foetal mass. Pregnancy can be first recognised via US by visualization of the embryonic vesicle (Ev) approximately by day 50 (Figure 43 - a), the presumptive yolk sac by day 75 (Figure 43 – b) and the zonary placenta by day 85. The trunk is first recognisable at 85 – 90 days into gestation (Figure 43 – c; Figure 44) and is distinct by day 104. The first heartbeats are evident from day 80 on (Figure 43 – f). By combining ultrasonographic and morphologic findings, the first reliable criteria for estimating gestational age and ontological development of Asian and African elephant foetuses was established, for the first third of the gestation (Figure 42, 43 and 44).

Figure 42: Schematic graphic of embryonic and foetal development from ovulation until birth. (Adapted from Drews et al., 2008)



* Embryonic period, ranging from 0 - 116 days post-conception.

** Just two allantoic compartments are presented in this figure, although a normal subdivision in four compartments is visible.

*** Time window for transrectal ultrasonography, ranging from 0 - 240 days post-ovulation.

Figure 43: Ultrasonographic stages during the first third of gestation (Hildebrandt et al., 2007). Scale bars – 1 cm

* 3D - Three-dimensional reconstruction

	Recognizable structures	Days of gestation
(a)	Embryonic vesicle (Ev)	50
(b)	Early embryo (Em)	74
(c)	3D* of an embryo; yolk sac (Ys), eyes (Ey), beak-like trunk (Tr), placenta (Pl)	97
(d)	3D* of an early foetus; physiological midgut herniation (Mh), right ear (Ea), typical trunk (Tr), one fore limb (Fl)	102
(e)	Echogenic plexus choroideus (Pc), trunk (Tr) positioned between the fore limbs.	108
(f)	Colour Doppler flow; foetal heart (He), arteria vertebralis (Av), umbilical vessels (Uv).	126
(g)	Foetus in lateral position	133
(h)	Allantoic pustules (AP) protruding to the allantoic cavity.	303

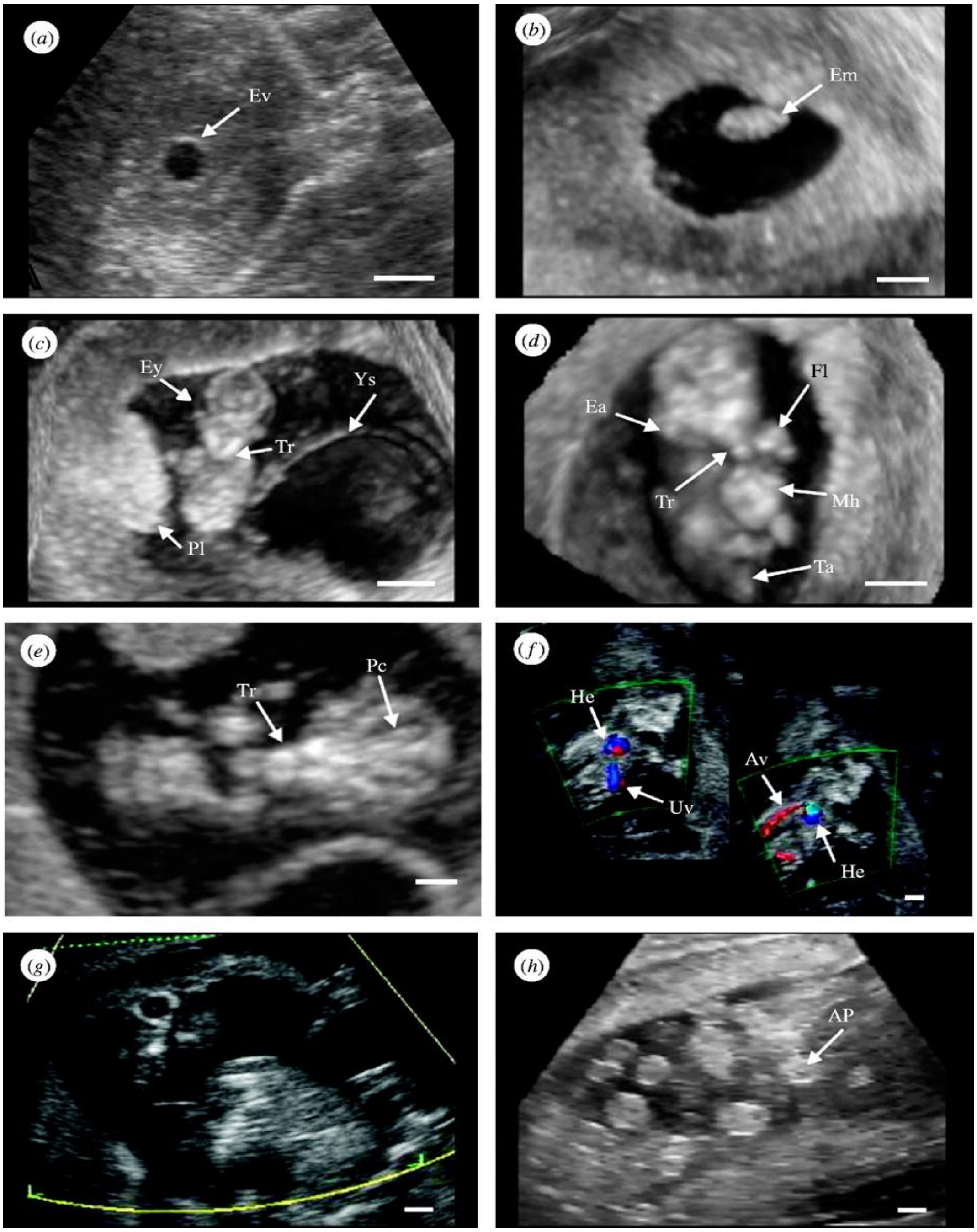
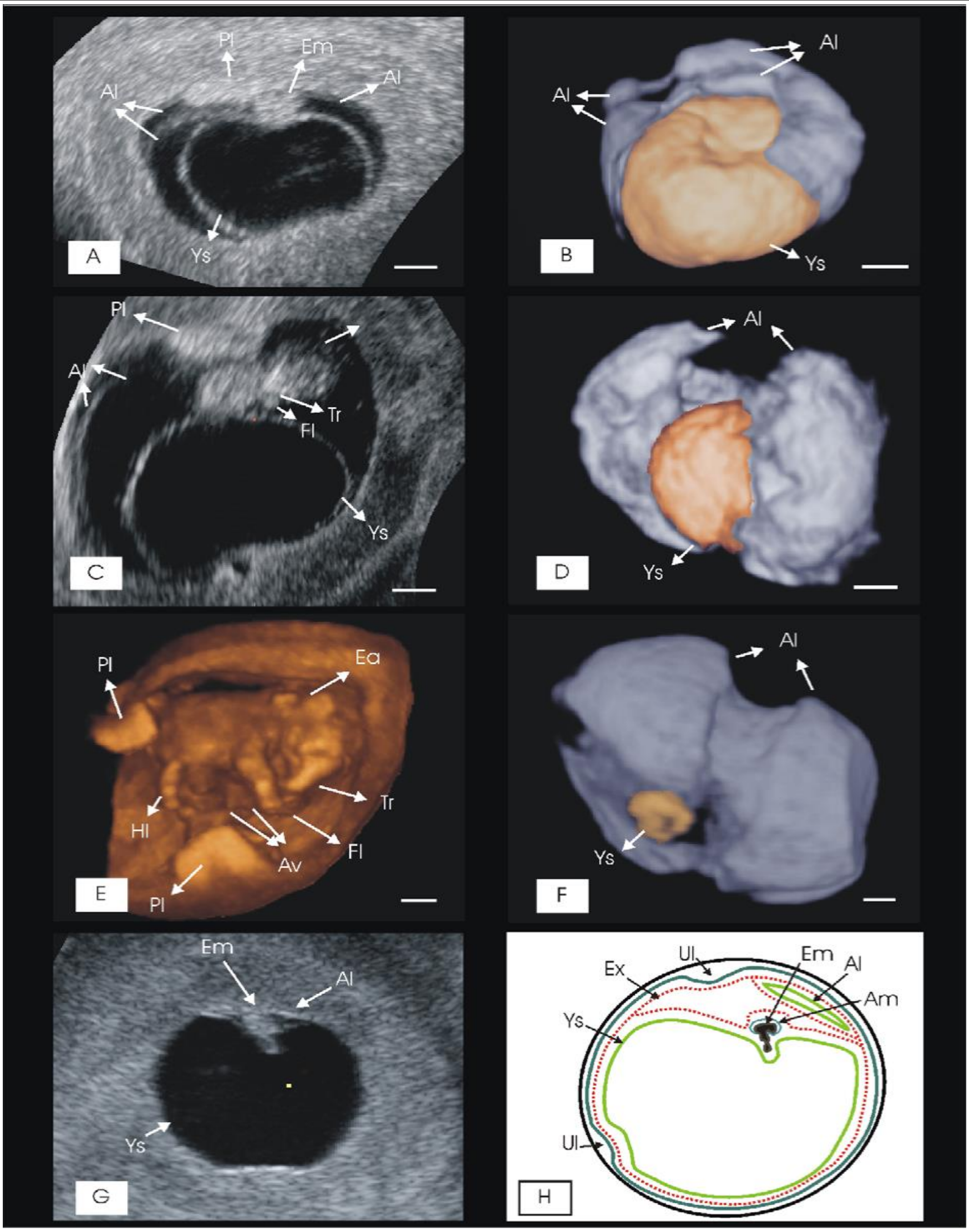


Figure 44: Ultrasonographic images of elephant conceptus (adapted from Drews et al., 2008). Scale bars – 1 cm.

	Recognizable structures	Days of gestation
A	Elephant conceptus: embryo (Em), chorioallantoic placenta (Pl), allantoic sacculations (Al) and Yolk sac (Ys)	83
B	3D in inverse render mode: allantoic sacculations (Al) and yolk sac (Ys).	83
C	Allantoic sacculations (Al), yolk sac (Ys), trunk (Tr), forelimb buds (Fl), allantochorionic placenta (Pl).	95
D	3D in inverse render mode: allantois (Al) and yolk sac (Ys); the allantoic sacculations embrace the embryo from lateral and dorsal.	95
E	3D in render mode of the foetus: typical elephant shaped trunk (Tr), ear (Ea), front (Fl) and hind legs (Hl), allantoic vessels (Av), placenta (Pl).	116
F	3D in inverse render mode: ring-shaped impression of the allantois (Al) – fully established chorioallantoic placenta; pedunculated yolk sac (Ys).	116
G	Sonogram of an Em, Ys and Al.	73
H	Drawing of an early conceptus: free uterine lumen (Ul), amnion (Am), allantois (Al), yolk sac (Ys). Mesoderm: red-hatched line, trophoblast: blue line, surrounding endometrial layer: black line.	≈75



4.6 Nutritional Management

A well-balanced ration should be provided throughout pregnancy for a normal foetal development. A total weight gain during gestation should be less than 250 kg in a mature cow (Schmitt, 2006).

Until late pregnancy there is little demand for nutrients for foetus growth, therefore a minimal weight gain is required for the cow during the first year of pregnancy, since at this time the combined total weight of the foetus, placenta, and foetal fluids is limited to around 30 kg. However, many captive elephant cows often gain weight significantly during this period. Overweight increases the risk of dystocia and stillbirth (Schmitt, 2006; T. Hildebrandt, personal communication, 2014).

Wild elephants spend much of their time (60-80% waking hours) feeding on a wide variety of low quality vegetation, including grass, browse, roots, fruit and flowers (Clubb & Mason 2012a). A dietary survey of European zoos revealed that elephants were deficient in various vitamins and minerals (e.g. zinc, iron, vitamin E), while 86.8% had a fat content exceeding recommendations. In another survey of European zoos, 90% did not provide grazing opportunities in the enclosure. However, the effect of diet on elephant welfare has yet to be assessed. Another study revealed that female zoo elephants were 31 to 72 % heavier than their wild counterparts, which is likely to be caused by the high fat content of the diets and a lack of exercise due to small enclosures and long periods of confinement and/or chaining. In the same survey, elephants that were regularly trained presented less of a weight problem than those that were not, but both were still heavier than elephants in range countries (Clubb & Mason 2012b).

Hay analysis can be very useful to evaluate its quality, and to design appropriate feeding programs. Poor-quality hay will have a good fiber percentage, but lacks protein. Furthermore, the mineral status concentrations, especially of calcium, can be low, and therefore may cause poor bone calcification. Additionally, it affects the blood calcium level, which may also lead to birth problems. A pellet with moderate amounts of neutral detergent fiber (NDF) and acid detergent fiber (ADF) is recommended for elephants. The normal diet should also contain high amounts of vitamin E and biotin to prevent muscle dystrophy and foot problems (Nijboer, 2015).

In summary, females should be monitored for weight gain during the entire pregnancy, and an active program is needed to stimulate the elephants to exercise. Feeding should be made attractive and food should be spread in different places of the enclosure, instead of feeding them once daily in the same routine spot, to help encourage the elephant to exercise. Also, a high variety of green food should be offered, and not just pellets. The lack of exercise and malnutrition should be prevented since it often leads to dystocia, foot problems and ventral oedema (T. Hildebrandt, personal communication, 2014).

During the nursing period, significant nutrient content is required by the dam to provide adequate milk for the calf (Schmitt, 2006). Proposed nutritional concentrations needed during breeding, early pregnancy, late pregnancy, lactation and growth of juveniles is presented in the Table 13 of Appendix I.

Calcium administration

The daily dietary calcium intake needed during pregnancy is still not well defined in elephants. However, it is likely that elephants follow the calcium metabolism of horses, i.e. being almost completely dependent on intestinal calcium absorption. It is known that insufficient milk production is an important cause of premature calf mortality in elephants. Extra calcium must be provided, since captive elephants are mostly fed poor quality hay with low calcium levels (Van der Kolk et at., 2008).

Based on extrapolation, calcium supplementation should be provided if ionized serum calcium (iCa) levels drop below 1.2 mmol/L (Van der Kolk et al., 2008). During parturition, calcium level must be closely monitored, and supplementary calcium administered (IV or orally) if the level decreases below 2.5 mmol/L for total calcium or below 1.2 mmol/L for iCa (Hildebrandt et al., 2006; Hermes et al., 2008; Schaftenaar & Hildebrandt, 2005).

For a good absorption of calcium, a sufficient vitamin D must be present.

It is advised that elephants should be exposed to UVB from sunlight; since it is suggested that oral supplementation of vitamin D in some cases is not sufficient (Nijboer, 2015). However, providing daily artificial ultraviolet light (290–319 nm) to increase vitamin D production is unfortunately not yet an option, as the destructive character of the elephant and a long trunk require a long distance to the device, resulting in an insufficient uptake of this potentially useful light source (Hildebrandt et al., 2006).

4.7 Pre-partum preparation

Arrangements with the staff and material acquisition should be organized several months in advance of the predicted birth date, as a 24-hour surveillance of the calf should be guaranteed, and as some of the supplies may require a special order and advanced shipment, as e.g. milk replacer (Emanuelson, 2006). Pre-birth preparations needed for an expected captive elephant parturition that are mentioned in the literature, are presented in Tables 5 and 6.

Table 5: List of the general supplies, medical supplies (which include many of those commonly found in a veterinary clinic) and some of the most important drugs to have available when expecting an elephant parturition (Original table. Information source: Olson, 2004d; Schaftenaar and Hildebrandt, 2005; Emanuelson, 2006).

General supplies	Medical supplies	Important drugs
- Cloth tape measurer, - Walk-on scale, - Video camera, still camera, record sheets, - Blankets, towels, baby wipes, shavings and straw, - For hand-rearing: bovine bottles and nipples, human breast pump, microwave, refrigerator, cooking thermometer, containers and utensils.	- Antibiotics, - Intravenous fluids, - Facilities for inhalation and injectable anaesthesia, - Endotracheal tubes (up to size 10), and equipment for oxygen therapy - Iodine solution for navel disinfection - Lubricant (many litters). - 20-30 mAmp portable radiograph unit (when possible).	- Ca-borogluconate for I.V. infusion, - Estradiol gel* - Oxytocin - Lidocain - Xylazine - Azaperone - Atipamezole - Doxapram

* Human gel Estrogel pump pack® (Hoechst) or Sandrena® (Orion Corporation).

A 24 hours' observation, including the use of a time-lapse video recorder, starting 5 weeks before the expected date, may add important information on relevant events prior to parturition (Schaftenaar & Hildebrandt, 2005).

Table 6: Pre-birth preparations needed for expected captive elephant parturition (Original table, Information sources: Olson, 2004d; Schaftenaar & Hildebrandt, 2005; Emanuelson, 2006; Hermes et al., 2008; Weber & Miller, 2012).

Pre-birth preparations	
Restraint of the dam	Check restraint chains and fixation points for the legs and one extra fixation point between the hind legs for pulling devices. Soft ropes for pulling the calf away if needed should be available. A calf harness may also be used.
Elephant plasma (4-6L)	Collected less than 6 months before the predicted birth date (from an elephant other than the dam).
Training of the dam	Desensitized the mother to gentle manipulation of the breasts and nipples, be careful not to massage excessively near parturition, which can reduce colostrum availability and may increase risk of mastitis and mammary gland oedema. Train for other veterinary interventions, such as blood sampling, injections, IV-infusions, milking and rectal manipulations. Especially important in nulliparous cows.
Milk replacer	Contact a nutritionist/specialist in hand-rearing elephants in advance is extremely important. So far, Salvana (GmbH, Germany) presented good results in hand-raising at Emmen Zoo and Berlin Zoo.
Calf – proof nursery	Nursery area in the elephant barn is necessary, including facilities for supplementary heating or cooling. Remove all obstacles and make sure there are no escape possibilities.
Absorbent material available	E.g., bran or sawdust – soaks up the amniotic fluids and provides better footing for the animals and keepers.
“Get a good grip”	Soccer gloves, soft woven cotton girth strap – useful to: 1. Support the calf until it is able to walk well, 2. Remove the calf faster if the cow becomes excited or aggressive.
Colostrum	Collect from dam after the birth, for future use, but only if the calf has ingested sufficient amount and the nursing process is not disrupted!
Rectal cleaning tools	Plastic hose pipe (with pump, if necessary) for rectal cleaning with lukewarm water.
Birth-chains	3 birth-chains with proper handles (2 for the legs, 1 for trunk or tail); find a way to avoid back sliding when manual extraction (vaginal vestibulotomy) is required.

4.8 Parturition

In most elephants, impending parturition is indicated by a precipitous fall in progestogens to base line levels, and birth usually occurs in the following 2 – 5 days. However, healthy calves have been reported to be born 7 – 12 days following this decline (Schmitt, 2006). Therefore, blood sampling for progesterone assays should be made every other day from week 89 until week 91, and daily from this point on, until a decrease in progesterone is detected. Then testing should be increased in frequency to twice a day (Schaftenaar & Hildebrandt, 2005). It is a myth that pluriparous elephants do not need to be monitored and assisted during labour, and many calves have been reported to born dead or very weak, due to unnoticed start and halt of parturition (Hermes et al., 2007).

Daily monitoring of progesterone is only possible if organized in advance with a nearby facility that runs these assays on a daily basis, and if this service is also available during the weekend (Schaftenaar & Hildebrandt, 2005).

A summary of the most common sings of impending parturition are presented in Table 7.

Table 7: Summary of impending parturition signs and behavioural manifestations (Schaftenaar & Hildebrandt, 2005; Hildebrandt et al., 2008).

Predicting the time of parturition, measures and observations	
Blood sampling for progesterone assay	Progestogens drop over a period of 1 – 2 days, usually to less than 0.10 – 0.15 ng/mL. Normally values decline to 50% in over 12 – 24 hour period, and then to baseline values within the next 12 – 24hours.
Loss of mucous plug	Vaginal mucous plug (15 x 10 cm) is a yellow-greenish sticky substance with traces of blood. Loss can happen within the last 2 weeks of gestation but usually a day before birth.
Ventral oedema	Before and after parturition a ventral oedema may be noticed.
Herd reaction	Herd members may react differently (e.g. vocalizations, restlessness).
Rupture of allantois sac	Reported in 75% of the cases; looks like urine and is seen up to 2 hours before birth.
Faecal balls size	Frequent production of smaller faecal boluses towards the end of pregnancy.
Urination frequency	Small quantities of urine may increase in frequency around parturition. Urine becomes more diluted*, resembling allantoic fluid.
Mammary gland changes	Emission of milk may occur near parturition; many cows produce milk only few hours prior to parturition, some only when labour occurs. Changes can be visualized using transcutaneous ultrasonography several hours prior to parturition.
Possible locomotion change	Softening of the pelvic ligaments (due to oestrogen surge) may result in slightly abnormal locomotion of the hind legs.
Changes in cow behaviour	Increased beating of the vulva with the tail, sudden “freezing” during movement, stretching, abdominal discomfort, refusal of normal diet.
Cervical dilation	Can be monitored by rectal ultrasonography.

* Due to this dilution, urinary glucocorticoids are unreliable for predicting parturition.

Ultrasonography is essential to determine foetal viability, cervical dilation, or presentation of the calf in the birth canal (Schmitt, 2006). If the calf is appropriately positioned in the birth canal, and if the cervix is dilated, oxytocin, vigorous rectal massage or both may stimulate labour. A large bulge underneath the tail usually becomes visible shortly before the calf is born (Hildebrandt et al., 2006).

To allow for birth, the umbilical cord (normal length 65 – 170 cm) needs to separate from the calf, as the path from the mother´s uterus to the vulva opening is longer than the length of the umbilical cord. This results in detachment and further expulsion. Like in other mammal species, the umbilical cord presents three blood vessels that retract into the abdominal cavity after birth (Schmitt, 2006).

Elephant cows give birth standing, so the calf drops to the floor and the amniotic sac usually breaks by itself. In the wild, the herd matriarch may be seen to push aside the new mother to watch over the new-born calf herself. In captivity, inexperienced cows are sometimes overly aggressive towards their offspring, and many facilities routinely restrain the dam during birth, allowing freedom to move, kick, and labour, but allowing for the new-born to be taken out of reach after birth until the mother’s behaviour toward the calf is assessed. This period also allows veterinary staff to evaluate the calf’s health (Wiedner, 2015).

The placenta is usually expelled within 4 - 12 hours after birth (although delays of several months have occurred) and the veterinarian should weigh and examine the placenta (Allen, 2006). Placenta retention has been reported to result in metritis (Lamberski et al., 2009).

4.9 Complications of birth

Dystocia occurs when a birth is considered abnormal or difficult to occur and the possible causes are listed on Table 8. To prevent or overcome dystocia in elephants, the staff team has to be prepared to invest in training and conditioning of the animal, training of the people involved, and to create the necessary infrastructure well ahead of the calculated parturition. It is extremely important to take into account this circadian rhythm of elephant labour activity; since elephant births occur mainly during the night or in the early morning, the team has to be prepared to work in night shifts. In elephants, dystocia is typically characterized by a normal start of the birthing process two days up to two weeks after the decline of the progestogens level to baseline, when a sudden interruption occurs. Sometimes, both the onset and the secession of labour pass unnoticed in elephants. This suspension of labour is reported to take place at any stage; after the mucus plug loss, or even after the rupture of the foetal compartments (Hermes et al., 2008).

Transrectal ultrasound is important in the evaluation soft tissue status in the pelvic birth canal, especially regarding the degree of cervical opening, which will allow an experienced examiner to diagnose a dystocia situation within 24 h (Hermes et al., 2008).

The best indicators for a developing dystocia are (Hermes et al., 2008):

1. Changes in the cow’s behaviour, presenting mental depression, night restlessness and loss of appetite;
2. Development of abdominal oedema;
3. Loss of interest in the birthing process by the other herd members;
4. Absent life signs of the calf during rectal palpation, external observation and/or transabdominal ultrasound examination.

Table 8: Possible causes of dystocia (Hermes et al., 2008).

Abnormalities of power (uterine contractions or maternal expulsive forces)	
Physiological uterine inertia	Exhaustion of uterine muscles. Dam ceases labour efforts due to pain, stress or other environmental disturbances.
Hypocalcaemia	Due to low levels of ionized calcium; ideally ionized calcium levels should be above 1.25 mmol/L when reaching labour;
Lack of physical fitness and excessive bodyweight	Especially in older primiparous elephants.
The passenger (viability, position, size, or presentation of the foetus)	
Intrauterine infection (can also be associated with death of the foetus)	EEHV infection is suspected to cause foetal death and stillbirth (in the Asian elephant population). Cowpox may cause calf death (essentially in Asian elephants). Salmonella is reported as a cause for foetal death (in African elephants).
Dead foetus by other causes	More prone to maloriention or malposition during labour. However, the elephant foetus can be retained for long periods of time (from months to 7 years), without causing any sickness in the cow, being later naturally expelled.
Overdevelopment or oversize calf	Calves over 120 kg and up to 200kg, probably due to an increased maternal nutrition program during pregnancy.
Abnormal presentation of the calf	Anterior presentation with abnormal head position; appears as a major cause of dystocia and a problem for foetal extraction; Twin pregnancy - simultaneous presentation of the calves, mutual interference or overly stretched uterine muscles; Foetal malformations - examples reported in elephants: encephalo-meningocoele, hydrocephalus, microphthalmia, spina bifida, cleft palate, missing maxilla and trunk, and teratology of Fallot; Arthrogryposis or ankyloses (Figure 46) - may be caused by maternal intrauterine infection, intoxication or oversized maternal tumours or cysts. These limit foetal movements, which may lead to dystocia and uterine rupture, resulting also in the death of the dam.
The passage (pelvis or soft tissues)	
Insufficient dilation of the birth canal	
Intact hymen	May obstruct the passage of the calf; bigger problem in older cows since the hymeneal tissue changes with time and the originally membranous structure becomes a more rigid septum.
Oedema of the lower reproductive tract (especially the vestibule)	Will narrow the birth canal and may suffocate the calf. Oedema develops long before the labour and preventive measures should be taken.
Urogenital tract pathologies	E.g., cysts, polyps, and tumours of the uterus, vagina, vestibule, and ovary. Extensive pathologies may fill the entire vaginal lumen and obstruct the passage of the calf.

The presentation of the calf in the birth canal can be determined by the direction of the toenails seen on rectal ultrasonography. Both anterior and posterior presentations are considered normal in elephants (Hildebrandt et al., 2006), although posterior presentation is the most predominant (Figure 45), and the most infrequent to cause dystocia in this species. It is hypothesised that it eliminates the risk of malposition of the head and gravity may help pulling the heavy anterior part of the calf into the birth canal in this presentation (Hermes et al., 2008).

Figure 45: Illustration of a pregnant African elephant presenting the foetus in the most common posterior position, inside the uterine horn (Image source: http://www.dkfindout.com/uk/animals-and-nature/mammals/mammals-and-their-young/).

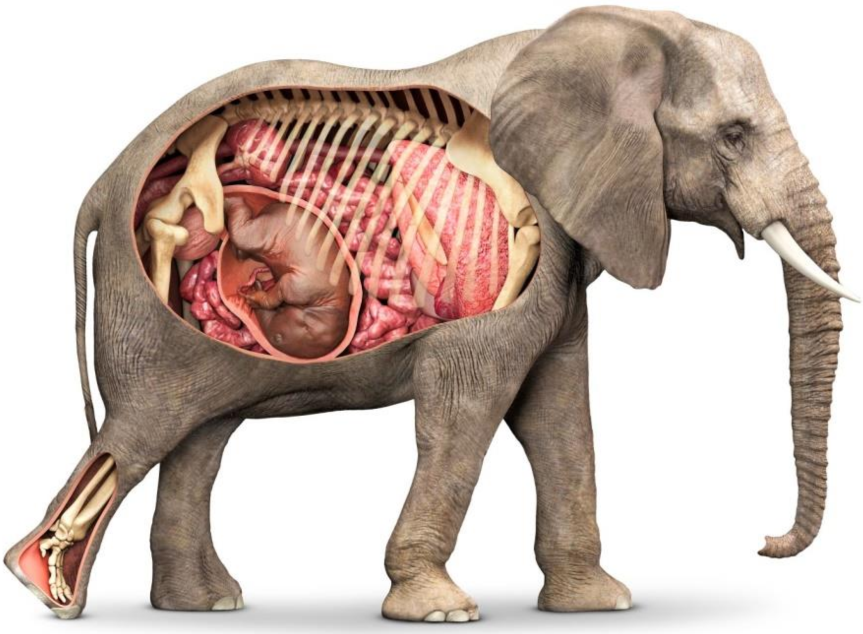


Figure 46: Malformation of the foetus presenting ankylosis caused by excessive abdominal lipoma of the cow, making this case impossible to deliver, not even with foetotomy (Hermes et al., 2008).

Possibilities for veterinary intervention during elephant birth

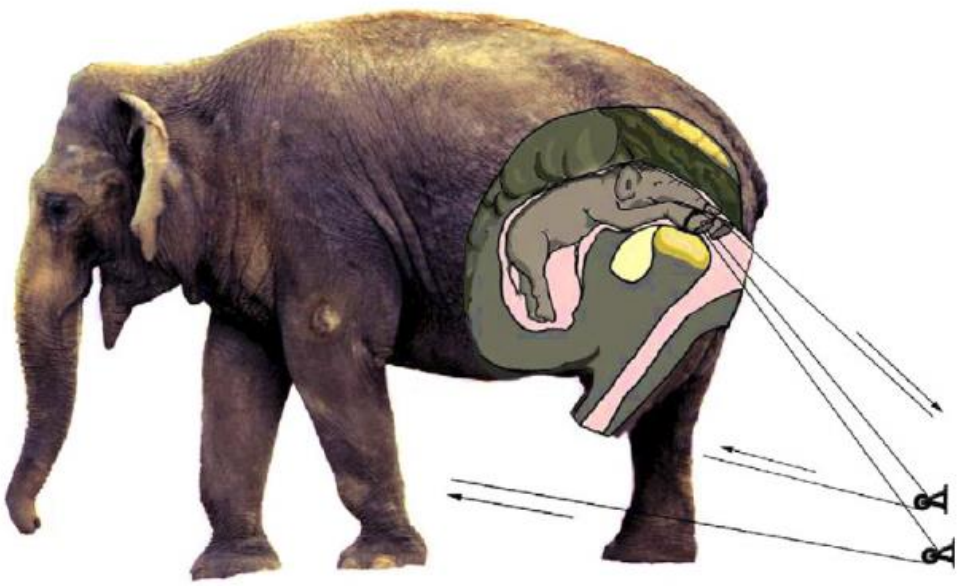
When progestogens drop below base-line levels and no visible signs of the onset of parturition are present within the next 24-48 hours, it is considered an abnormal condition, which requires veterinary intervention (Schaftenaar & Hildebrandt, 2005).

Veterinary intervention options during an elephant birth or dystocia differ greatly from any domestic species and are far more limited, mainly due to the dimensions and specific anatomy of the elephant and the reproductive tract. Caesarean section is the common last resource in domestic animal obstetrics, however is unsuccessful in elephants. Therefore, non-invasive medical treatment is of great importance in elephant obstetrics. For example massaging the uterus, inducing the Fergusson reflex, making the animal exercise, or simply leaving a retained foetus until it is expelled voluntarily are some of the non-surgical decisions (Hermes et al., 2008).

Surgical approaches such as episiotomy or foetotomy is sometimes inevitable to save the life of the mother, however, they normally result in chronic post-surgical complications or fatal outcome (Hermes et al., 2008). Vaginal vestibulotomy is a surgical procedure, in which the vertical part of the urogenital tract (vestibulum vaginae) is exposed by a percutaneous approach (Figure 47). The technique is indicated if no progress in calving is observed, despite given treatment around parturition, or when malposition is noticed. It is however contraindicated when no foetal parts can be detected in the birth canal (Schaftenaar & Hildebrandt, 2005).

The entire vaginal vestibulotomy procedure in elephants is described in the veterinary guidelines by Schaftenaar & Hildebrandt (2005) and will not be detailed in this work.

Figure 47: Surgical extraction of foetus during episiotomy, with detail on the direction of the ropes during forced extraction (Hermes et al., 2008).



Chapter 5 Neonatal Care

5.1 The newborn elephant

Elephant calves present a low survival rate in comparison with other captive mega-vertebrates. In a neonatal healthy program is intentioned to keep the elephant calf with the mother, and in case of forced separation, all efforts should lead to reintroduce both together again (Emanuelson, 2006). It is extremely crucial to develop a plan for the reintroduction of the calf to the dam and birth preparations should include preconditioning the female.

Regarding the newborn calf, a plan for 24 hour care should be developed with antecedence, and the fact that elephant calves quickly bond to their caretakers should be taken in account. Therefore, the team should be a consistent small group, and the calf should not be allowed to bond with just individual since may can lead to separation anxiety by the calf. Emanuelson (2006) advices that two calf caretakers should be all time with the calf, which should never be left alone.

Immediate separation from the mother after parturition is indicated in case of (i) a nulliparous mother, (ii) a dam with a history of aggression towards calves, (iii) dystocia, or (iv) an undersized or weak calf, (Emanuelson, 2006; Weber & Miller, 2012). Although, in case of an apparently healthy calf is born to a healthy multiparous female, with no background of aggression to her calves, is recommended not to interfere during the initial neonatal period and bounding. Examination should be performed just after nursing, if the assess do not disturb maternal bond (Emanuelson, 2006), during the first 24-48 hours (Weber & Miller, 2012).

If separation is necessary, the calf should be carried just a short distance away from the mother, in order to allow visual contact, smell, and touch but prevent grab, step on or harm for the new-born or the staff. This allows the cow to recover from the birth and gives the opportunity to perform a neonatal exam and administer any necessary medication, while providing time for the calf to stand. Aggression from the mother to the new calf tends to occur when the calf struggles to rise and vocalizes. Therefore assisting the calf may help prevent this negative reaction. If necessary the cow should be sedated to allow the calf to nurse during this critical period (Emanuelson, 2006).

5.2 Neonatal examination and care

Most calves are able to sit in sternal position 5 - 10 minutes after birth and to stand within 10 - 30 minutes. Slightly cyanotic membranes might be present initially, becoming pink within 10 - 20 minutes. Normally the urine and meconium will pass in the first 6 hours of life and after this moment calves typically urinates a minimum of twice a day and produce faeces at least once a day (Wiedner, 2015). After 2 days of age without passage of the meconium, pathology should be suspected – e.g. congenital defects such as atresia ani or coli, constipation, dehydration, gastrointestinal stasis or insufficient milk ingestion (Weber & Miller, 2012).

Umbilical infection is a major cause of perinatal complications in elephants (Emanuelson, 2006). The umbilicus usually breaks very close to the calf's abdomen and dries up over 24 to 48 hours. Often, a considerable swelling around the umbilicus remains for the first few days of life. Although considered normal, a variety of umbilical problems have occurred in elephant calves, including hernias, fistulas, patent urachus and prolonged bleeding from umbilical vesicles. Thus, the abdomen should be visually evaluated and palpated and ultrasonography performed, if needed. If umbilical bleeding appears excessive, suturing may be necessary (Wiedner et al., 2008). Both surgical and nonsurgical management of umbilical hernias are possible (Abou-Madi et al., 2004; Wiedner et al., 2008).
Immediate examination of calf should assess several health values and umbilical treatment should be applied (Table 9).

Table 9: Initial after birth and ongoing ideal calf health examination (Emanuelson, 2006; Weber & Miller, 2012; Wiedner, 2015).

Assessment of calf health		
Umbilical stalk treatment	1x Initial treatment with tincture of iodine	Minimize risk of infections, accelerate closure and drying of the stalk.
	4x in first day with diluted chlorhexidine solution	Treatment may need to be done while calf is sleeping or dam is chained to reduce discomfort or stress to the calf.
	3x daily for 3 days with chlorhexidine solution	
Body weight/height	Daily/when possible - for the first 2 weeks	Average calves birth weight: 110 kg (53-153) for L.africana 100 kg (50-148kg) for E.maximus Calf should gain 0.45 – 1,4Kg/day in the first year. Approximated birth height of 0,91 m.
Oral examination	2x/day	Check for ulcers, swelling, cyanosis (late signs of EEHV infection in most cases).
Respiratory rate (monitor trends)	2x daily	At birth: 60 – 80 breaths/min; Fast decline to 30 – 50 breaths/min, slower when sleeping; 1-week old: 20-22 breaths/min.
Heart rate (monitor trends)	2x daily	At birth: 60 – 90 bpm 1-week old: 115 (100-128) bpm
Rectal body temperature (monitor trends)	2x daily	Normal range: 36.3 – 37^{o}C
CBC and Chemistry panel	(if indicated)	Range values in Wiedner (2015).

bpm – beats per minute, x – time(s).

A normal calf is active, vocal, and curious about its environment. Calves that deviate from normal behaviour even in minor aspect should get a thorough medical evaluation (Wiedner, 2015).

5. 3 Nursing

Suckle reflex is usually present at birth, and nursing should start in the first 2 – 12 hours (in few calves can go up to 24h without any ill effects) (Wiedner, 2015), in order to receive the full amount of colostrum, ranging around 2 – 10L (Emanuelson, 2006). Approximately 74% of calves attempt to nurse in the first 7 hours of life (Weber & Miller, 2012). A stool may be used to help a small calf to nurse (Figure 48).

Milking the elephant cow may be possible by hand (Figure 49) or using a human breast pump (Figure 50) and it is possible to obtain 300-1000 mL/milking. To facilitate the procedure oxytocin (30-60 UI IM) may be administrated previously (Weber & Miller, 2012).

Figure 48: In case of a very small calf/very tall dam, a stool may be used to help nursing (Image source: Olson, 2004b).

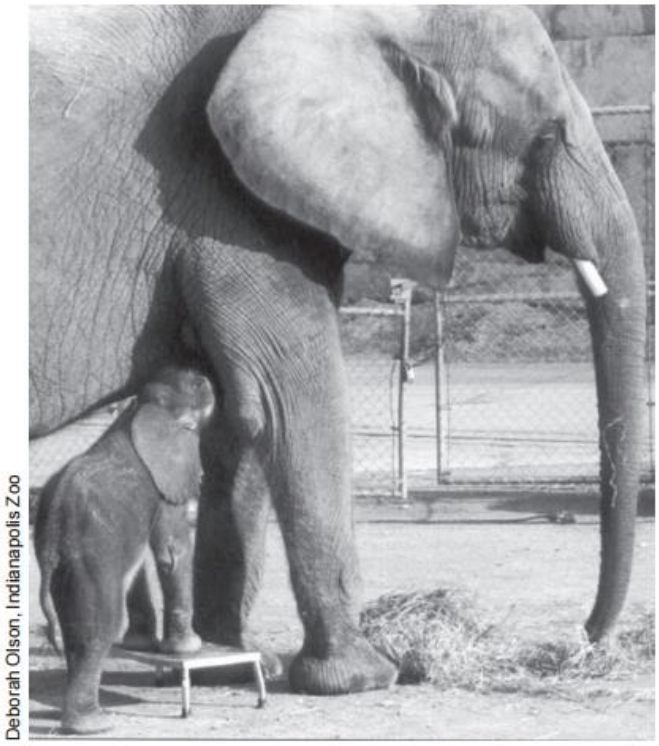
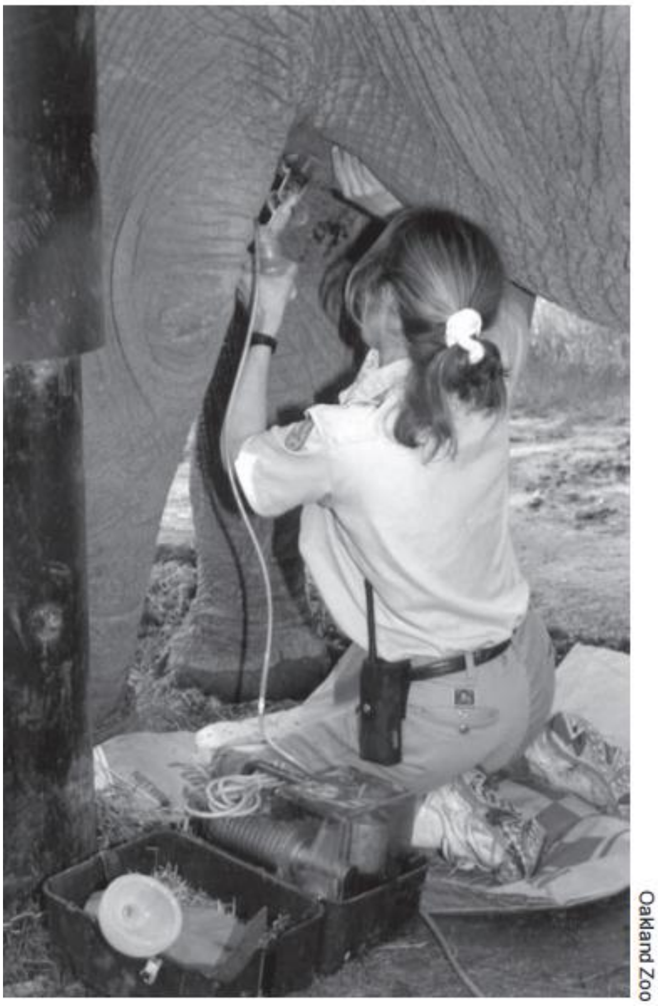


Figure 49: Milk collection by hand and desensitization of the mammary gland and nipple manipulation in order to stimulate the calf's initial attempts to nurse and milking (Olson, 2004d).

Figure 50: Electric human breast pump being used to collect milk from an elephant cow (Olson, 2004d).



5.3.1 Failure of passive transfer (FPT) of antibodies

In case the calf does not nurse from the dam within the first 12 – 24h, supplementation should be considered with colostrum from the dam, milk from the dam or replacer, or plasma. Supplements may be offered with bottle and nipples used for bovines, or forced via stomach tube. Milking the colostrum from the mother, or feeding banked elephant plasma or commercial equine hyperimmune plasma may also provide oral immunoglobulin supplementation. This will provide the local immunity in the intestine even when antibodies are no longer absorbed. Plasma transfusions may present a solution in more severe situations as for example weak calf, at risk of infection). A total of 40-80 mL/kg IV over a period of 2 - 4 days or a single bolus may be given in 30-60 min. Total protein and gamma globulin levels and tests for FPT should be repeated after the transfusion (Weber & Miller, 2012).

5.3.2 Hand-rearing

Creating appropriate and adequate formulas for orphaned calves has been a challenge; the available commercial formulas have required supplementation with vitamins and fats and have been associated with diarrhoea and inadequate weight gain in several cases. Raising orphans or rejected neonates have been very difficult (Wiedner, 2015). A proposed nutrient composition for hand-rearing elephant calves can be found in Table 10.

Table 10: Hand Rearing nutrition required for zoo animals specifically elephant calves, proposed by MERCK veterinary manual. (Source:http://www.merckvetmanual.com/mvm/management_and_nutrition/nutrition_exotic_and_zoo_animals/handrearing_zoo_mammals.html?qt=elephant&alt=sh).

Nutrient Requirements for hand-rearing elephant calves, ruminants, pigs			
Dry matter (%)	Protein (%)	Fat (%)	Carbohydrates/Lactose (%)
12-23	21-27	30-45	20-37

5.3.3 Weaning

Weaning is a gradual process, and involves both modification of the internal anatomy and alteration of the microbial flora within the intestines. This intestinal flora is obtained when the calves consume fresh faeces of the adults. Calves are introduced to solid food by sampling food dropped by the mother within the first few months and are consuming some plant material by 6 months of age. The elephant management staff, with input from the veterinary care stuff, will assess the best age to wean a particular calf based on the health of the calf, its personality, and the mother's behaviour toward it. An indication that calf may be ready for weaning is when the calf has a faecal consistency of an adult. The mother may also indicate the best time to wean a calf by an increasing lack of tolerance toward it. Most elephant breeders believe weaning is best to occur between 2 and 3 years of age. Separation and weaning of the calf can be done in many different ways, but it is labour intensive, needs to be properly planned, require continuous evaluation of the process, and demand good facility design and competent staff (Olson, 2004a). A detailed table with growth weights of 11 elephant calves, from birth until 5 years of age, can be found in Olson (2004a).

5.4 Training

Initial training should begin shortly after birth. It is a gradual process, but it is important that the calf learns to interact with humans and every interaction allows the handler to mold and encourage desired behaviour, in order to facilitate husbandry and medical care. The calf should be taught to tolerate medical examination, blood collection, foot care, treatment of injuries, trunk wash for tuberculosis testing, and daily mouth examination. It is also important for the calf to be conditioned to accept some limited separation from its mother. The training session should be kept short, end on a positive way, and be fun for the calf (Olson, 2004a).

Chapter 6 Lisbon Zoo

6.1 Lisbon Zoo herd reproductive situation

Lisbon Zoo has a management of “Protected contact” policy for interaction with the *L.africana* herd; meaning that the keepers have contact with the elephants through a protective barrier of some type, being the elephant free to leave the “working” area at any time.

The Zoo current elephant herd is constituted by 1 male – “Jasa” and 5 females – “Jane”, “Nina”, “Luna”, “Primavera” and “Assunção” (Table 15 in Appendix IV). The females can be separated in 2 age groups; 3 individuals around 23-24 years being considered proven breeders adults, and a younger group, around 8 years of age with 2 juvenile females with unknown reproductive status. The male is a young adult and with no offspring.

To access the breeding capacity of “Jasa” a semen collection trough manual rectal stimulation was attempted on November 2013. Unfortunately, the procedure was interrupted due to safety issues. Besides a sufficient standing sedation, it was not possible to locate the male elephant into a safe working area, where the animal could be secured with additional foot chains. The anaesthesia report and the sedation used during the procedure can be found in the Appendix II and Table 14 of Appendix III.

Since 2003, 4 births occurred in Lisbon Zoo. The first and second parturition unfortunately resulted in calves that died with 5 days and 3 years of life, respectively. With the third and fourth births, in 2005 and 2006, 2 juvenile females were born and are living currently in Lisbon elephant herd.

The successful management of the parturitions called “Group Birth Approach” allows the dam to be always in the herd, with permanent separation of the breeding bull. All four births occurred in the outside enclosure, although access to inside enclosure was granted. After the birth event, dam and calf were separated in the inside facility.

6.2 Lisbon Zoo reproductive recommendations

Analysing the current breeding situation at Lisbon Zoo, IZW reproductive management recommendations are to examine the older females, in order to evaluate the genital tract status and their current breeding potential.

It is mandatory to prevent long inter-birth periods, since like previously explained, can cause several reproductive pathologies and can lead to complete infertility and severe health issues, such as periodic blood loss from the reproductive tract, foot problems due to accumulation of toxic factors (e.g. tumour necrosis factor) or severe abdominal pain demonstrated by periodic colics.

Due to the young age of the male comparing with the older elephant cows, there might be a situation of domination by these females. Therefore, an exchange with an older and more experienced, proven breeding bull should be considered, or the time gap between Jasa's reproductive maturation and now should be overbridge by sending temporarily the reproductive assessed females to another breeding facility or by considering artificial insemination under standing sedation.

It is considered of extreme importance to prevent long periods of senescence for the younger females, which present already around 8 years of age, a time near conception for their wild counterparts. These females should be allowed with natural mating opportunity, and a continue observation of the interest presented by male on copulating with them is needed.

On a personal communication with Dr Rui Bernardino, the senior staff veterinarian of Lisbon Zoo, the following recommendations were approached and discussed, and are hereafter presented.

Currently, all females show regularly and in synchronization, signs of oestrous. The elephants are not trained for veterinary medical procedures, therefore, hormonal monitoring is performed sporadically in faeces. Cycling females are detected by observation of heat manifestations.

The male "Jasa" arrived in 2011 and since then, attempts of copulation were observed with a particular juvenile – "Assunção". Recently, in the last 6 months, these attempts also embrace the adult female "Nina". All of these mating attempts correspond to mounts without observed penile penetration.

The possibility of an artificial insemination program and the necessary requirements for such approach was strategically discussed with the responsible zoo staff and Dr Thomas Hildebrandt.

A training program was implemented for the elephants, during 2 years with the support of Mr Alan Roocroft, and results were highly positive. At the moment, however, the training was interrupted due to several reasons.

Concluding, on the point of view of breeding management, changing "Jasa" for a more experienced and proven breeder bull would makes sense, despite being always dependent on the availability of a proven breeder, and the transportation costs have also to be taken in account. At the moment, considering the young age of the individual and the ongoing adaptation process of a male that did not received the learning needed in a group, Lisbon Zoo is positive on the possible developments, based on the recent evolution of his breeding behaviour, that Jasa will be successful in is natural copulation progressive attempts.

Chapter 7
Clinical Cases in elephant Reproduction

MATERIAL AND METHODS

The following clinical cases were gathered during reproductive examinations performed by Dr Thomas Hildebrandt. The author of this work had the opportunity to assist the veterinary procedures during the cases discussed below by scanning the females' reproductive tract, diluting the freshly collected semen in prepared warmed tubes, analysing the ejaculate, preparing all the necessary equipment for the visit and further transportation of the semen samples, helping in the collection of sonographic data during the examinations, among other tasks. The main focus off these reported cases was to gain a deeper understanding of how reproductive management aids conservation efforts in captive elephants.

1 Subjects

Four captive elephants were assessed; two males and two females. All animals were scanned with ultrasound technology to examine the genital tract. Following the sonography, a semen collection was performed in the two bulls. Identification, general information, and reproductive anamnesis of the four individuals are detailed below.

1.1 "CALVIN"

Calvin was a Asian elephant bull, 27 years old at the time of the following reported visit. He was born in captivity at Calgary Zoo (Canada) in 11-08-1986, was transferred to African Lion Safari (Canada) in 03-04-1989, to Hannover (Germany) in 16-03-2000, to Leipzig Zoo (Germany) in 11-09-2008, and finally to Ostrava (Czech Republic) in 12-05-2009 (http://www.elephant.se/database2.php?elephant_id=212).

Reproductive anamnesis

From 1998 until 2014, Calvin produced a total of fourteen offspring; nine males and five females. Four calves were born in Canada, three in North America - one of which produced with assisted reproduction by AI technique (Brown et al., 2004) - and seven in Europe (Table 11).

Calvin was considered a good semen donor since the first reproductive examination and semen collection, presenting always an average sperm concentration of $6x10^8$ to $1x10^9$ sperm cells per mL. The bull was a proven breeder in the breeding EEP program for the Asian elephants since 1998 (T. Hildebrandt, personal communication, 2017).

Table 11: Identification of the previous offspring from the bull “Calvin” (adapted from http://www.elephant.se/database2.php?elephant_id=212).

Sex	Name	Date of birth	Place of birth	Date of death	Reason of death
M	Samson	1998-05-04	African Lion Safari, Canada		
F	Annie	1998-07-30	Have Trunk Will Travel, US	1999-03-22	Haemorrhagic pulmonary oedema
M	Amos	1998-08-21	Have Trunk Will Travel, US	2002-08-08	Intestinal torsion
M	Albert	1998-11-29	African Lion Safari, Canada		
M	George	1999-10-21	African Lion Safari, Canada		
M	Johnson	2001-04-29	African Lion Safari, Canada		
M*	Kandula	2001-11-25	Smithsonian National Zoological Park, US		
F	Califa	2003-02-02	Hannover Zoo, Germany		
F	Farina	2003-03-20	Hannover Zoo, Germany		
M	Tarak	2005-10-28	Hannover Zoo, Germany		
M	Shanti	2008-05-06	Hannover Zoo, Germany		
M	Sethi	2011-03-11	Ostrava Zoo, Czech Republic	2011-05-06	Unknown
F	Rashmi	2011-04-12	Ostrava Zoo, Czech Republic		
F	Sumitra	2014-02-04	Ostrava Zoo, Czech Republic	2016-01-23	EEHV**

* Conceived by AI
** Elephant Endotheliotropic Herpesvirus

Reason for examination

The bull was accessed due to the need of viable sperm to perform an AI in an Asian elephant female in Kolmarden Safari, Sweden. The female was known to be ovulating the day of the collection, since her hormonal profile was being monitored during all oestrous cycle.

Other information

Calvin was euthanized in 22-10-2015, with 29 years of age (around two years after the described examination), due to severe feet problems; after a poxvirus infection that damaged his ligaments, walking became heavily defected due to a deformed hind foot. The animal could still mount and reproduce, but during time it lead to foot problems in the front feet, ending with the loss of one front toe bone, and being euthanized due to extreme pain.

The dams of Annie and Amos were transported in 1995 to African Lion Safari (Canada), staying for 3 years for natural mating until pregnancy was achieved. In 1998 they both returned to Have Trunk Will Travel (US), to give birth to Calvin’s offspring. Although still in practice, transportation of animals (especially females) is becoming a less desired solution to improve the breeding efforts of the elephant captive populations.

1.2 "TEMBO"

Tembo is an African bush elephant bull, 32 years of age at the time of the reproductive evaluation. He was born in free range (wild) in 1983 in Zambia, arrived in 1984 to Longleat Safari Park (UK), and was moved to Mary Chipperfield (UK) at an unknown date. The animal was later rescued, arriving at Colchester Zoo (UK) in 28-02-1998, where he lives nowadays.

Tembo is a famous elephant, unfortunately not for the best reasons; he suffered from abuse at Mary Chipperfield, a circus that was convicted for animal cruelty. However, after being recovered by Colchester Zoo, in 2001 he was father of the first European artificially inseminated calf – Abu (http://news.bbc.co.uk/2/hi/uk_news/258191.stm; http://www.telegraph.co.uk/news/worldnews/europe/austria/1330131/The-baby-elephant-thats-just-made-history-in-Vienna.html).

Reproductive anamnesis

Tembo was father of eight European offspring: four males, with three calves produced with the use of AI, and four stillborn with undetermined gender, all from the same mother – Zola (Table 12).

Table 12: Identification of the previous offspring from bull "Tembo" (adapted from http://www.elephant.se/database2.php?elephant_id=6).

Sex	Name	Date of birth	Place of birth	Date of death	Reason of death
M *	Abu	2001-04-25	Vienna Zoo, Austria		
M *	Kito	2002-12-06	Colchester, UK	2017-03-12	Heart failure
Unknown	-	2003-12-10	Colchester, UK	2003-12-10	stillborn
M	Jambo	2004-03-15	Colchester, UK		
Unknown	-	2005-04-05	Colchester, UK	2005-04-05	stillborn
M *	Thabo-Umasai	2006-02-04	Dresden, Germany		
Unknown	-	2008-03-01	Colchester, UK	2008-03-01	stillborn
Unknown	-	2011-10-08	Colchester, UK	2011-10-08	stillborn

* Conceived by AI

The bull was part of the EEP breeding program for African elephants during many years and used as a semen donor for several AIs. In the past eight years he started presenting subfertility problems; producing ejaculate samples with a sperm concentration between aspermic and $15x10^6$ sperm per mL, and since then no more calves were born.

It is also believed that his estimated age was not accurate, and the bull might be older than previously expected (T. Hildebrandt, personal communication, 2017).

Reason for examination

The reproductive management team was present for Tembo's annual check-up, which is highly recommended by the breeding program for African elephants.

1.3 "DELHI"

Delhi is an Asian elephant female, 32 years of age at the examination, and was wild born (unknown place) in 1983. There is no data on how long the animal stayed in Saigon Zoo (Vietnam) before being sent to Usti ned Labem Zoo (Czech Republic) in 17-06-1987, the place she lives nowadays (http://www.elephant.se/database2.php?elephant_id=1629).

Reproductive anamnesis

Delhi was previously part of the Asian elephant breeding program and was artificially inseminated in 2002. Although pregnancy was attained, the dam presented dystocia, stopping labour efforts at term, and retained the dead foetus for seven days. She gave birth to a male stillborn and oversized calf (160kg) in 2004-07-01. During this one week of delate birth, the treatment involved several massages everyday with estradiol and prostaglandin E2 gel, transrectally. After this episode the female failed to get pregnant again, stopped cycling and presented a fluid filled uterus.

Reason for examination

This individual was accessed for an annual check-up, to examine the state of cyclicity to attempt AI once more.

1.4 "KALA"

Kala is an Asian elephant female and was 30 years of age at the examination. The female was wild born in 1985, arrived at Usti nad Labem Zoo (Czech Republic) in 30-11-1985, after being transferred from Saingon Zoo (Vietnam) (http://www.elephant.se/database2.php?elephant_id=1627).

Reproductive anamnesis

Kala never had any offspring, and presented reproductive tract pathologies at the first reproductive examination. The animal was diagnosed with endometrial tumours (leiomyoma) with associated external bleeding. Therefore, she started therapy with GnRH vaccine using Improvac® (Zoetis Deutschland GmbH, Berlin, Germany), a GnRH synthetic analogue, as an attempt to treat/reduce her lesions. Kala is under treatment for two years, with an administration of 2 mL (300µg) every six months.

This female is not considered a suitable breeder and is not part of the Asian elephant breeding program.

Reason for examination

An annual examination was made to check possible progression of the reproductive pathologies that could compromise the health of the animal and to verify if the GnRH vaccine controlled the bleeding situation and its effects on the regression of the reproductive lesions.

Delhi and Kala are the only two Asian elephants at Usti nad Labem Zoo (Czech Republic), having no access to males for natural mating. Both males have access to herds with potential breeding females in their holding Zoo.

2 HANDLING AND RESTRAINING

The females Delhi and Kala have a management policy of "Free Contact" (Figure 54), which means that the animals can be accessed without any physical separation from the keepers. In case of the males, they are handled in "Protect Contact" (Figure 53 and 56).

All of the following described procedures - rectal ultrasound examination and semen collection - were accomplish without administration of sedatives. For the female reproductive check-up, positive reinforcement was present by feeding fruits and vegetables during all the procedure. All animals were scanned in standing position, and the females were also assessed in lateral recumbence, demonstrating the importance of a good training program for positive cooperation of the animals. During the examination and semen collection of Tembo and Calvin, the bulls were restrained standing in a chute, with the four limbs chained (Figure 55).

3 TRANSRECTAL SONOGRAPHIC EXAMINATION OF THE REPRODUCTIVE TRACT

3.1 Equipment

All ultrasound examinations were performed using Voluson i (Voluson i, GE Medical Systems, Zipf, Austria), a portable ultrasound system, that provides 2D and 3D views of the reproductive tract. A 2 to 5 MHz transducer probe was used, in both male and female, with a specific customized probe extension for elephants – Figure 51 and 52.

Figure 51: The ultrasound examinations were performed with "Voluson i" and an abdominal probe of 2-5 MHz (Original picture).

Figure 52: Extension used to access the four animals, measuring approximately 45 cm. The US probe is adapted to the extremity of the extension (Original picture).

3.2 Ultrasound scanning technique for males and females

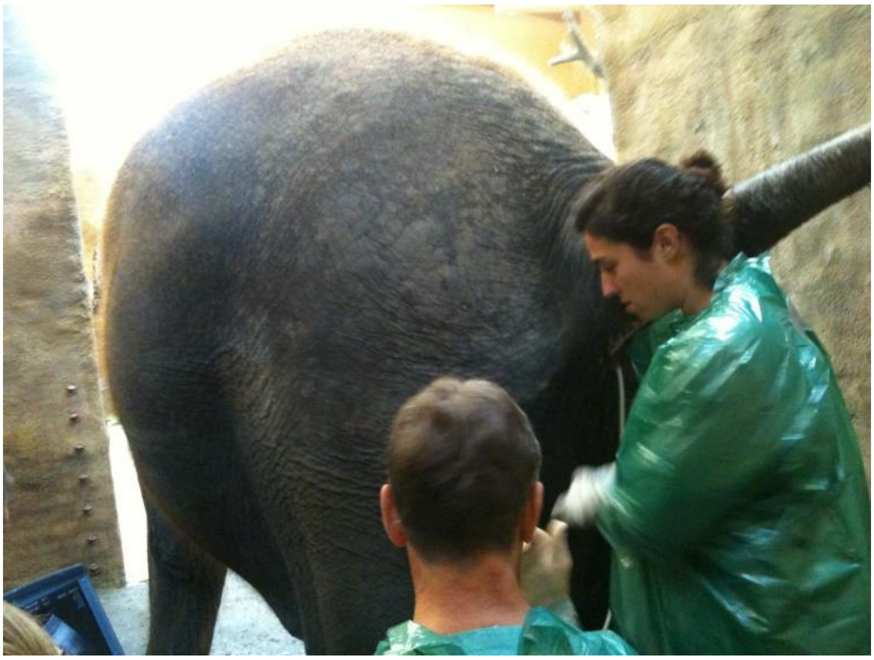
Before each examination faeces were removed by manual cleaning and irrigation with lukewarm water (Figure 53). The probe, hand and arm of the operator were lubricated, an essential step to prevent mucosa damage and improve the interface between the probe and the structures. The ultrasound probe was then manually guided over the reproductive tract by rectal acess. Normally, during the ultrasound, scanned structures were not immediately measured, with the exception of follicles and possible pathological findings (e.g. cysts, tumours), to reduce as much as possible the duration of the exam.

For the females the transducer was used to visualize the caudal region of the genital tract: vestibule, urethra, vagina, urinary bladder, cervix and caudal part of the uterine body. To access the cranial region of the reproductive tract, the extension was adapted so that the cranial part of the uterine body, uterine horns, ovaries and surrounding tissues could be scanned.

The bulls were examined for all genital structures with the exception of the testicles, as the animals were being examined without sedation. Due to their internal position (2 meters from the rectal access), scanning this gonads risks causing pain to the animal, which is also dangerous to the US operator, and might compromise the semen collection.

Figure 53: Manual removal of faeces, using a hose without sharp endings and presenting a small diameter (1 – 2 cm). Note the need of help from the keepers to maintain access to the rectum in the bull, handled in “Protected contact” (original picture).

Figure 54: Ultrasound examination on Delhi, in a “Free Contact” system, with no sedation and in standing position (original picture).



4 MALE REPRODUCTIVE EVALUATION AND SEMEN COLLECTION TECHNIQUE

During all the procedure, keepers talked to the bulls in a calm deep voice to help reduce stress.

Semen was collected using manual stimulation trough the rectum. A rectal massage was applied on the pelvic portion of the urethra (near the seminal colliculus), stimulating the penis to protrude and accomplishing erection (Figure 55). The penis was then cleaned and dried, to reduce contamination of the semen sample. A collection sleeve (condom) is then placed underneath its tip (Figure 56 and 57). With the stimulation of the pelvic urethra, an ejaculatory response was detected, directing the massage also to the *ampulla* region of the ductus deferens until the ejaculation was obtained. The semen was collected in fractions to avoid possible urine contamination and the condoms were changed between each fraction.

Figure 55: Erected penis achieved by rectal manual stimulation. Note that the four limbs are chained and the bull is restrained standing in a chute (original picture).

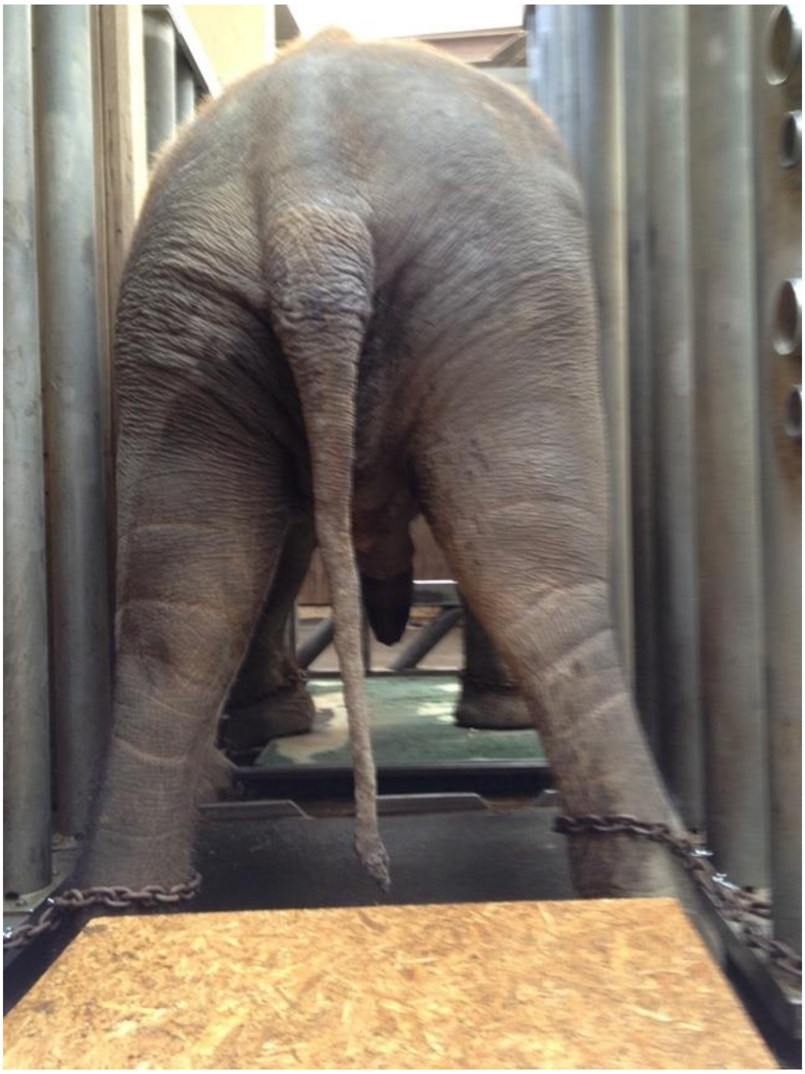


Figure 56: Exteriorized penis with condom for semen collection, during the rectal manual stimulation approach. Note that the keeper holding the device is physically protected from the bull (Original picture).

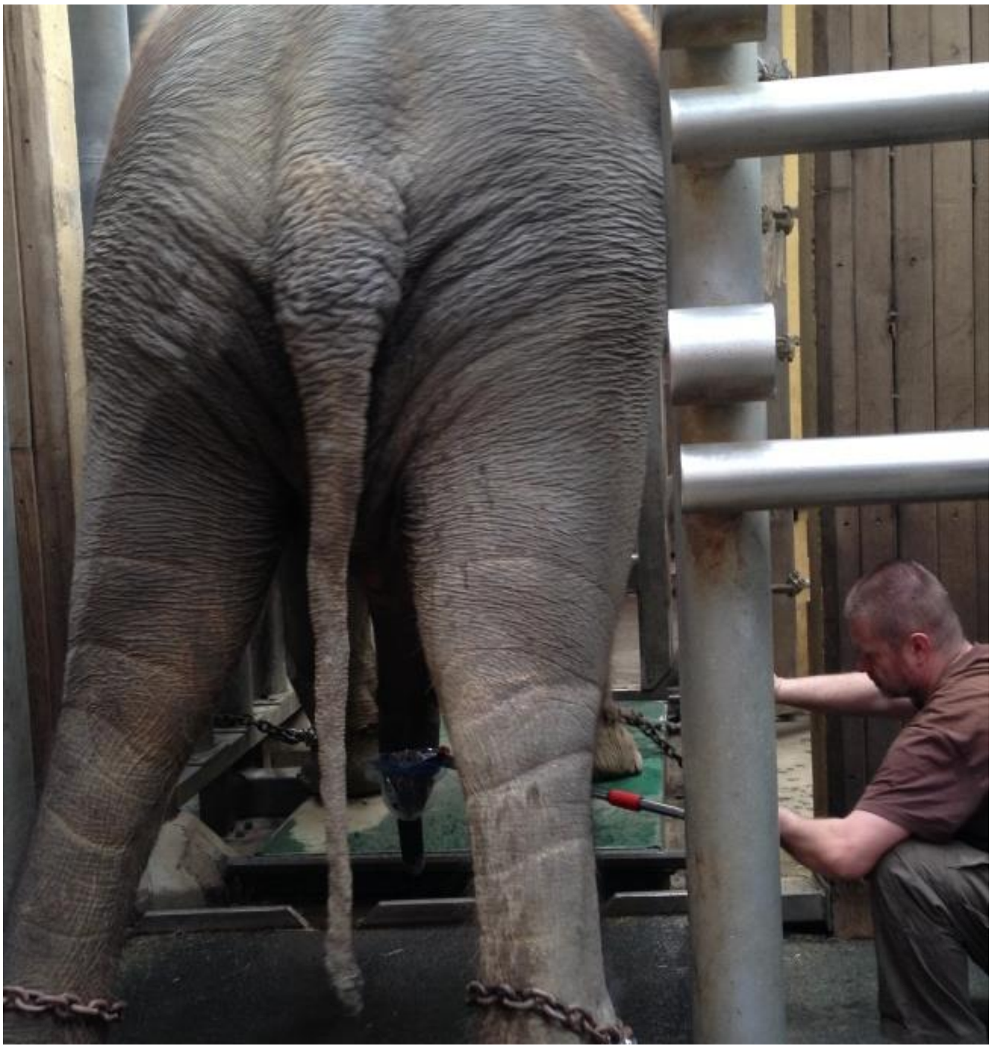


Figure 57: Semen collection device - sleeve (condom) to place underneath the penis tip. A clean plastic bag will funnel the semen into a 50 mL tube. The bag is adapted to the end of a pole/extender, to lengthen the safety distance from the animal (note: the bag should reach the entire surface of the metal ring) (original picture).

Immediately after collection, the condom was removed and the sample was labelled. Each fraction was assessed for volume and sperm motility (using a light microscope).

The semen samples were then diluted in a proportion of 1:1 with Berliner Cryomedium kept at 37^{o}C (previously described in Chapter 3). When extended, the semen presented a temperature of 35-37^{o}C, being then cooled at 4^{o}C in a portable semen cooling chamber (EQUITAINER®; Hamilton-Thorne, Beverly, MA) for shipment (Figure 58).

Once arrived at the destination, around 30 minutes before the AI, the semen was warmed in a water bath of 37^{o}C and assessed for sperm motility (T. Hildebrandt, personal communication, 2017).

All instruments were thoroughly clean and disinfected after each examination.

Figure 58: Device commonly used at IZW to transport fresh semen to be used for AI – EQUITAINER® (original picture).

RESULTS

Results will be presented for each animal, by reporting the initial ultrasound examination, followed by the evaluation of the ejaculated for the males and finally, decisions derived from all the findings.

1 Reproductive examination findings in the males

1.1 “CALVIN“

Transrectal ultrasound assessment

Calvin ultrasonographic findings are indicated in Figure 59. All of the organs appear with a normal structure. The *ampulla* was filled with a “snowy” fluid, without separation of density, which can be seen moving at the US examination, representing that the sperm is alive (Figure 59– D). The seminal vesicle in this bull was not extremely full (folds indicate that it’s just partially filled) (Figure 59– E and F), which indicated that the testosterone levels were not very high (no measurements of this hormone were made at the time of the examination).

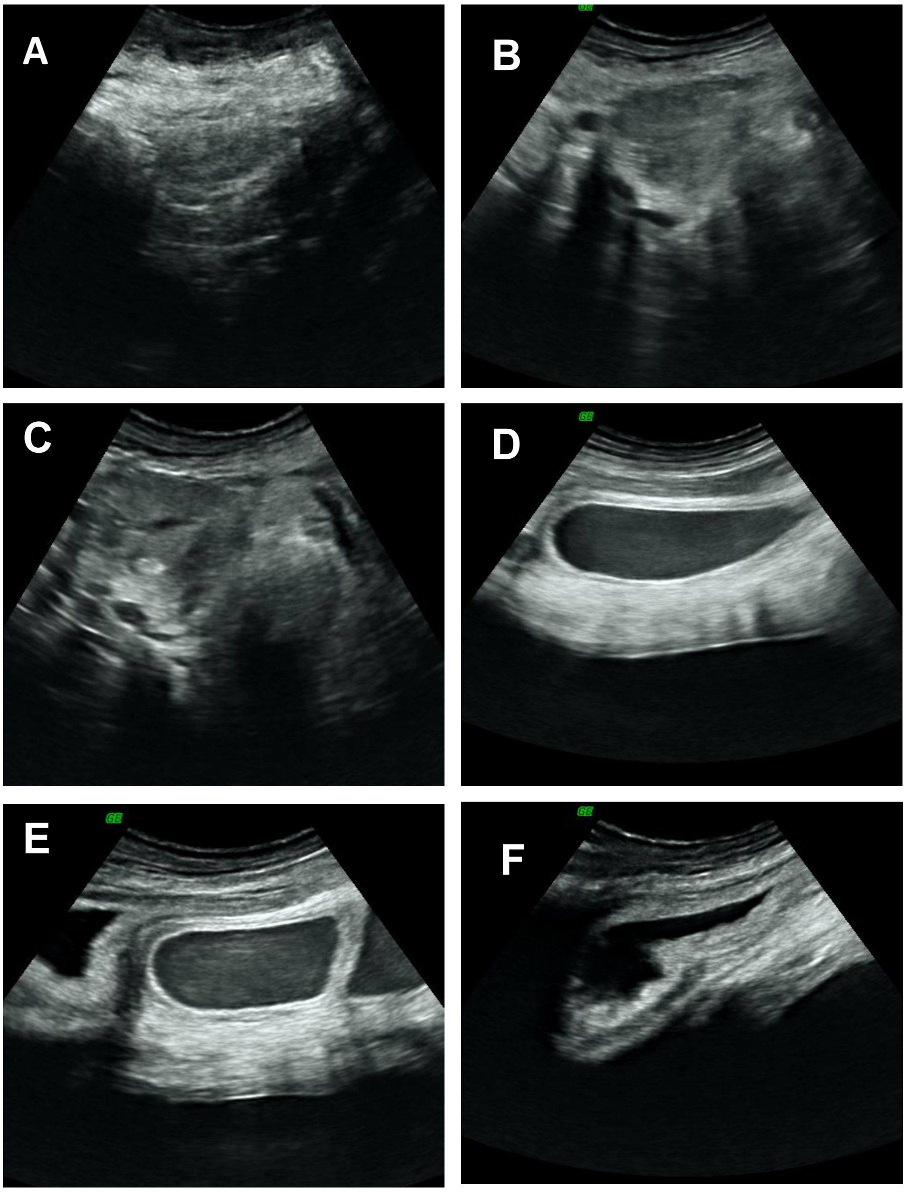
Ejaculate characteristics

Analysing the semen samples, we obtained three fractions of ejaculate, in a total volume of 18 mL, all presenting a white colour. The sperm had a motility higher than 90% and approximately 80% of normal sperm morphology (by observation).

The semen was stored at 4˚C in a EQUITAINER® device (Figure 58) and transported to Kolmarden Zoo (Sweden). There, a 19-year-old female – Sanoi – was under hormonal monitoring, and was about to ovulate. The insemination took place around 12h after the semen collection. The semen presented a motility of 65% after warmed to a temperature of 35 degrees.

After Calvin’s semen was collected and freshly preserved, one AI attempt was made within 12 hours in an Asian female that was ovulating at the time. Unfortunately, the female did not get pregnant from this intervention, but she is still part of the breeding program of her species, and future AI is expected.

Figure 59: Ultrasonographic finding of the reproductive tract examination of the bull “Calvin”: A – urethra; B – prostate with visible lobes (left side); C – blood vessels, lobes, and bridge, creating a “butterfly” sitting in the urethra with *cuniculos seminalis* inside (left side); D – Left *ampulla* filled with snow fluid; and ureter entering the bladder (in cross-section); E – Both *ampullae* are visible and the left seminal vesicle can be seen; F – Left seminal vesicle presenting folds.



1.2 “TEMBO”

Transrectal ultrasound assessment

Ultrasonographic images of the reproductive examination of Tembo are presented in Figure 60. The urethra had a normal appearance (Figure 60 - A), the prostate presented no content but the lobes were easily recognized (Figure 60 - B and C). The *ampullae* were empty, presenting no evidence of fluid content (Figure 60 - D, E, F), however it was possible to visualize the seminal vesicles nicely filled (Figure 60 - G, H). The seminal vesicle and the bladder presented deposits, visible by their hyperechogenicity (I and J).

Ejaculate characteristics

Tembo's semen was collected in four fractions, with a total volume of 200 mL. The samples presented a yellowish colour and were sperm free (aspermic).

When comparing colourations of the different ejaculates, Calvin presented a white coloured sample, and Tembo's was yellowish, which indicated urine contamination, or lack of good semen quality and quantity.

Figure 60: Ultrasonographic findings of the reproductive tract examination of the bull "Tembo": A – urethra; B – prostate without content, visible lobes (right side); C – empty prostate (left side), D – empty *ampulla* and bladder (right side), E – empty *ampulla* with seminal vesicle on top (right side); F – empty *ampulla*, seminal vesicle, urethra (left side); G – seminal vesicle nicely filled (right side); H – seminal vesicle (left side), I – seminal vesicle with deposit of protein (right side); J – bladder with hyperecoic deposits, visible blood vessels.

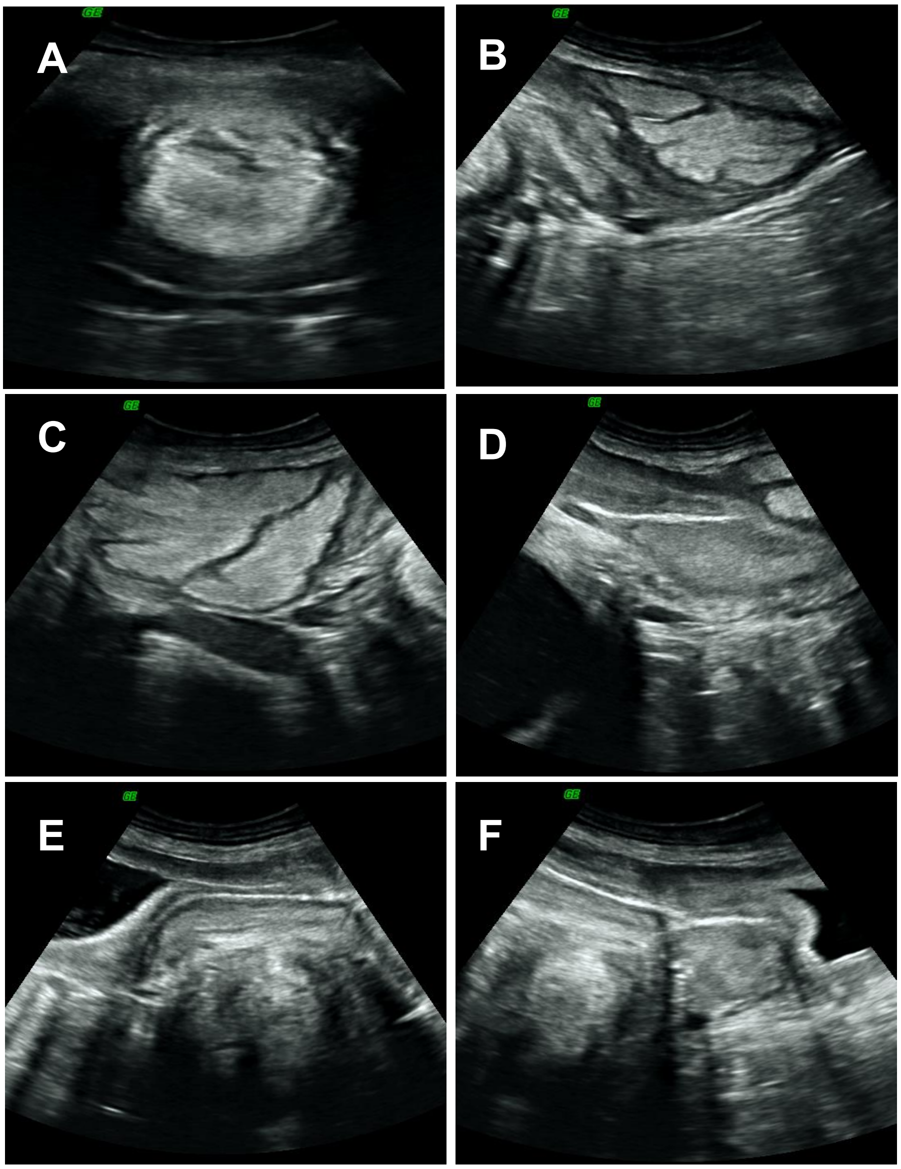
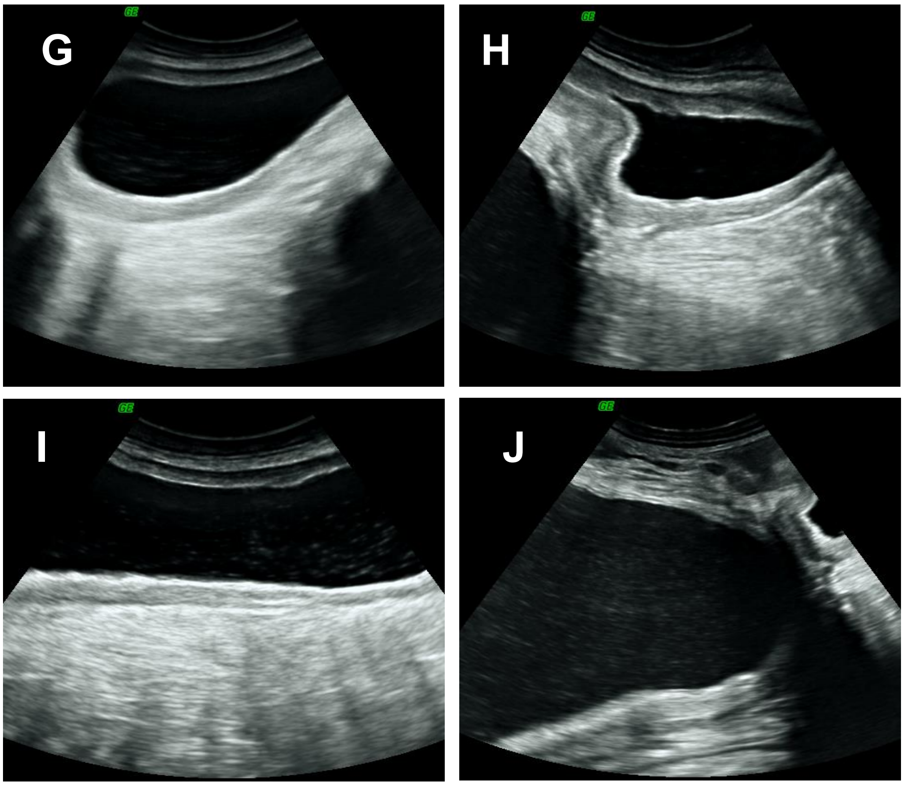


Figure 60 (continuation): Ultrasonographic findings of the reproductive tract examination of the bull "Tembo": A – urethra; B – prostate without content, visible lobes (right side); C – empty prostate (left side), D – empty *ampulla* and bladder (right side), E – empty *ampulla* with seminal vesicle on top (right side); F – empty *ampulla*, seminal vesicle, urethra (left side); G – seminal vesicle nicely filled (right side); H – seminal vesicle (left side), I – seminal vesicle with deposit of protein (right side); J – bladder with hyperecoic deposits, visible blood vessels.



2 Reproductive examination findings in the females

2.1 "DELHI"

Transrectal ultrasound assessment

Ultrasonographic images of the reproductive examination of Delhi are presented in Figure 61. The vestibule is shown with no alterations (Figure 61 – A). The urethra presented a calcification in its caudal part (Figure 61- B). The vagina appeared with a small mucous volume, and the left ovary presented a small follicle in development measuring 0.99 cm in diameter (Figure 61 - C and I). In the uterus, endometrial cysts were visible (Figure 61 - J). The contralateral ovary presented an old CL (Figure 61 - E and F) and a cyst measuring 2.18 cm in diameter (Figure 61 - G).

Figure 61: Ultrasonographic findings of the reproductive tract examination of the female “Delhi”: A – vestibule, in its horizontal portion (cross section); B – urethra presenting a stone/calcification; C – vagina (longitudinal view) with small mucous volume at the lumen; D – uterine endometrium with its visible coiled arteries (arteria spiralis); E – right ovary; F – right ovary with an old CL; G – ovarian cyst of 2,18 cm in diameter (right ovary); H – tip of uterus in cross section; I – left ovary with a small follicle of 0,99cm (in diameter); J – uterine endometrial cysts.

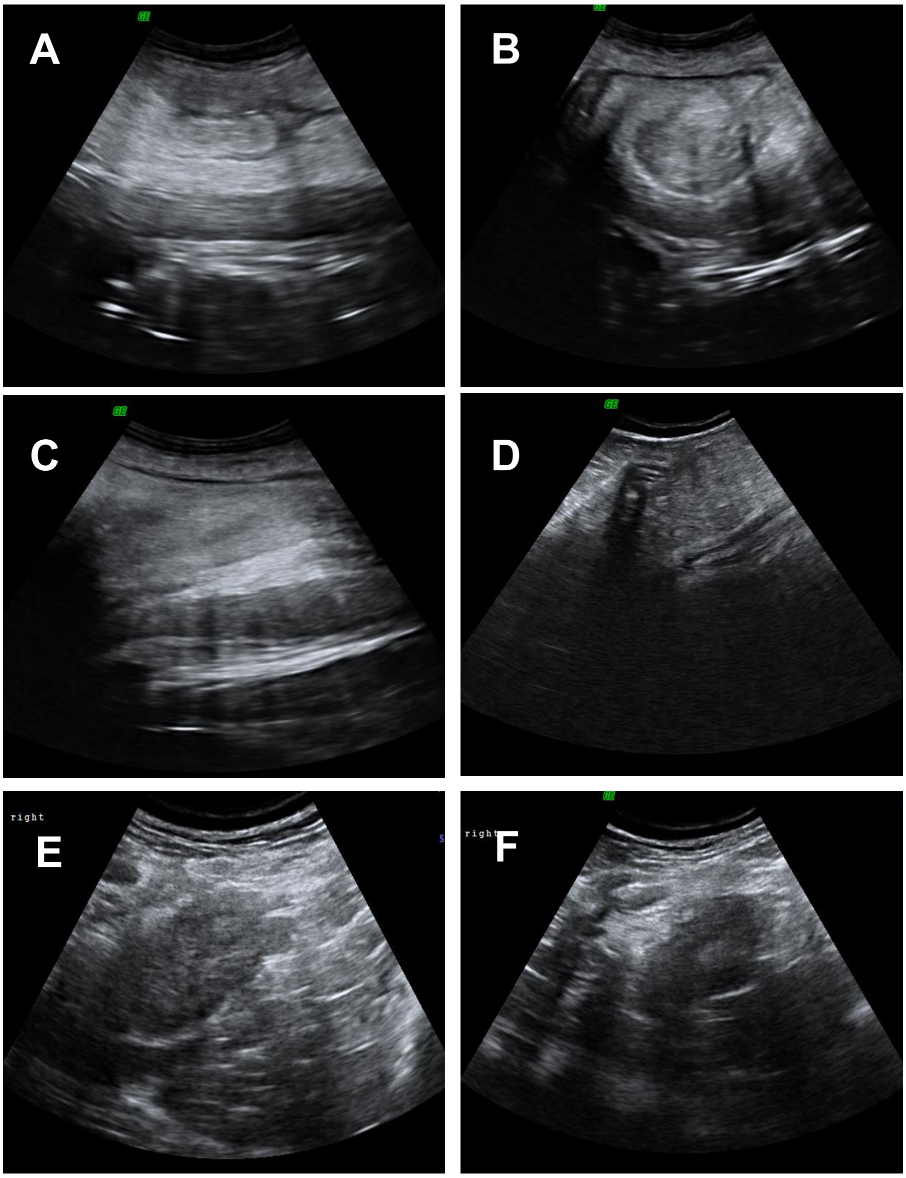
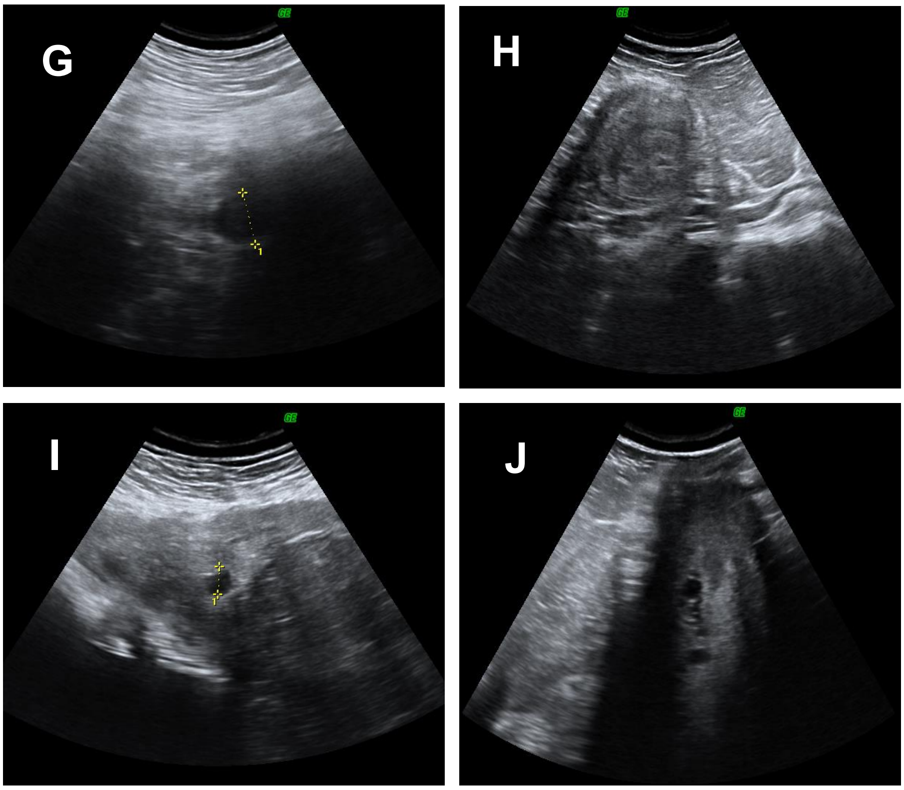


Figure 61(continuation): Ultrasonographic findings of the reproductive tract examination of the female “Delhi”: A – vestibule, in its horizontal portion (cross section); B – urethra presenting a stone/calcification; C – vagina (longitudinal view) with small mucous volume at the lumen; D – uterine endometrium with its visible coiled arteries (arteria spiralis); E – right ovary; F – right ovary with an old CL; G – ovarian cyst of 2,18 cm in diameter (right ovary); H – tip of uterus in cross section; I – left ovary with a small follicle of 0,99cm (in diameter); J – uterine endometrial cysts.



2.2 “KALA”

Transrectal ultrasound assessment

Kala’s ultrasonographic findings are shown in Figure 62. The urethra presented a stone at its caudal portion (Figure 62 - B and C), but the bladder had clear urine (Figure 62 - D and F). The vagina presented a sticky mucous white line (Figure 62 - E and F) and cysts (Figure 62 - G and H). Cystic structures were also found in the fornix (cercyst) (Figure 62 - I and J), in the cervix itself (Figure 62 - J) and in the endometrium, creating a “cheese like” appearance (Figure 62 - K). The endometrium presented also a leiomyoma (Figure 62 - K). In the right ovary a big sized ovarian cyst is visible, with a diameter of approximately of 3,5 cm (Figure 62 - L).

Figure 62: Ultrasonographic findings of the reproductive tract examination of the female “Kala”: A – vestibule, with a visible muscular part; B – stone in the beginning of the urethra and several blood vessels; C – longitudinal view of the urethra with calcification; D – vagina with a white line in lumen, bladder with clear urine; E – vagina, presenting with white line; F –mucous in the vagina and the detail of the ureter entering the bladder; G – cyst in vagina; H – same; I – cyst in front of the fornix, and another cyst in the cervix; J – cercyst; K – cheese like endometrium (cysts) and leiomyoma; L – ovarian cyst.

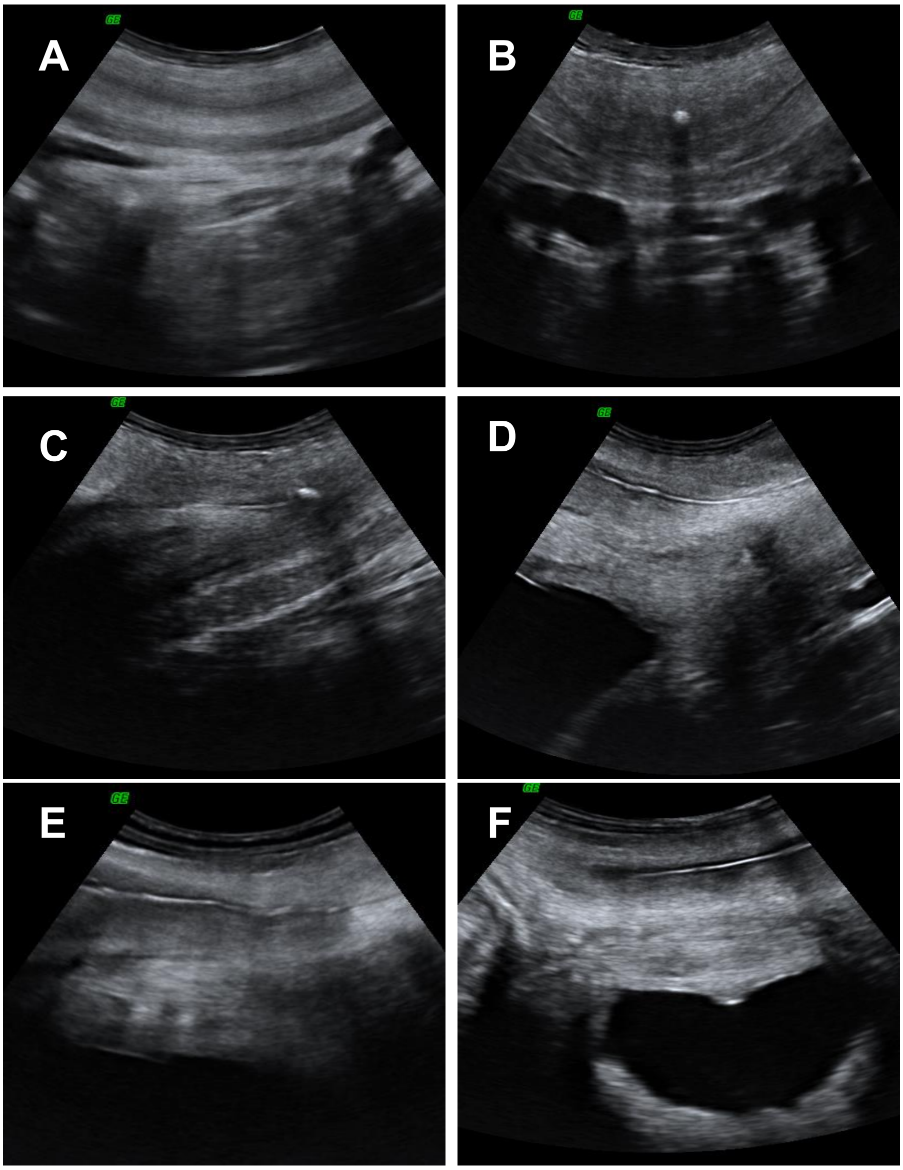
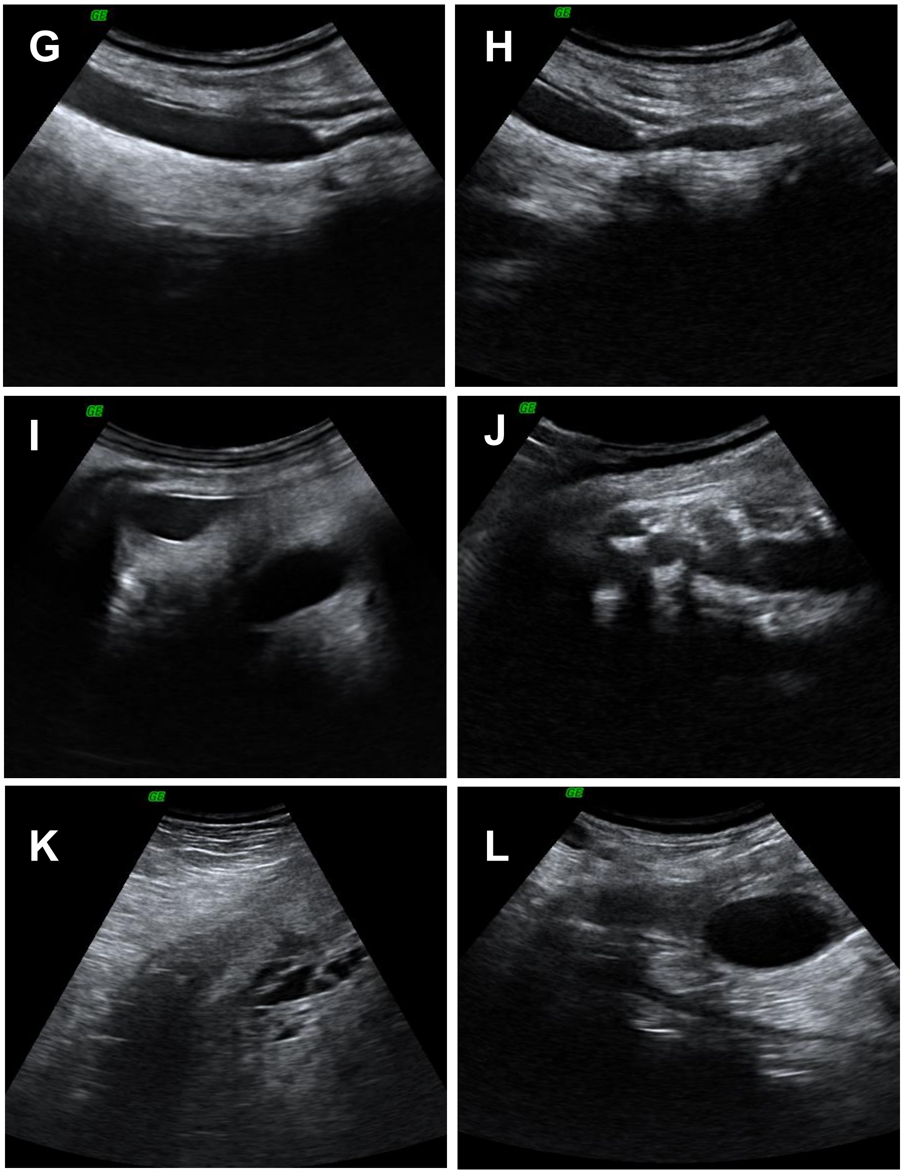


Figure 62 (continuation): Ultrasonographic findings of the reproductive tract examination of the female “Kala”: A – vestibule, with a visible muscular part; B – stone in the beginning of the urethra and several blood vessels; C – longitudinal view of the urethra with calcification; D – vagina with a white line in lumen, bladder with clear urine; E – vagina, presenting with white line; F –mucous in the vagina and the detail of the ureter entering the bladder; G – cyst in vagina; H – same; I – cyst in front of the fornix, and another cyst in the cervix; J – cercyst; K – cheese like endometrium (cysts) and leiomyoma; L – ovarian cyst.



Although the female still presented the previous pathologic findings, and no regression in its dimension was seen, the bleeding had stopped after the first administration of Improvac®.

DISCUSSION

Although representing specific clinical cases, the results obtained from the examinations of these four individual help enlighten the current state of reproductive management performed in captive elephants.

From the three bulls that the author had the opportunity to follow and are described in this work, just one presented good fertility parameters, one was aspermic, and in one was impossible to collect a semen sample to achieve an understanding of his breeding status (continuing as a possible breeder, but not yet a proven breeder). The examined females present several pathologies, were incapable of breeding and one had a history of dystocia.

Regarding the used methods in these clinical cases, the transrectal ultrasonography is the standard and most suitable technique for evaluation of the reproductive tract in elephants. A good training will reduce stress associated with the technique and, in particular with elephants, cooperative animals of several tons are crucial.

Manual stimulation through rectal access is the most commonly used technique in elephants to obtain semen samples. The use of this approach to achieve semen presents advantages when compared with electroejaculation and artificial vagina methods.

This technique allows fractionation of the ejaculate into sperm rich fractions, while other collection techniques do not usually provide this option. The use of electroejaculation often results in samples with gelatinous accessory gland contamination, and requires general anaesthesia. Disadvantages for the use of AV are the need of extensive and time consuming training and the habituation to the device (Schmitt & Hildebrandt, 1998).

For better results, training of the animals for cooperation with this veterinary intervention is of particular importance. However, if needed, the holding Zoo is responsible for the sedation of its' animals, with the success being dependent of age, previous attempts, individual metabolization of the drug, among other factors. The most common drugs used for adult sedation in bulls is a combination of Detomidine with Butorphanol (Appendix III, Figure 63).

For all the performed procedures in the addressed reproductive clinical cases, the use of no sedation or reversal antidote drugs, made it safer for the animal's health.

Reproductive findings in males

Although several analogies are made between African and Asian elephants, it is important to remember that species specific differences exist. In the results obtained for the urethra, the ultrasonographic image revealed that the urethra in Asian elephants is not as prominent as that found for the African elephant (compare Calvin and Tembo US findings). The prostate presented also anatomical differences, as previously mention in Chapter 2, between the two species. It was also possible to visualize that the Asian elephant (Calvin) presents a smaller sized prostate when compared with African elephant (Tembo). However, it is not comparable in its structure, since Tembo presented a prostate without internal cavities (Figure 60 B and C), resembling more a normal functional prostate of an Asian elephant (Figure 59 B and C), being considered a negative indicator of inactivity for an African elephant.

For both bulls a complete procedure of semen analysis was not performed, being only accessed for volume and sperm motility. Different reasons lead to this; Calvin was previously known as a good semen donor, and the ejaculate analysed parameters were in his normal average, plus the short timing for preforming the AI was an important factor to trust his reproductive examination with the information already gathered (full *ampulla*, great sperm mobility), and for Tembo, since no sperm cells were collected, no further analysis could be made to the ejaculate.

There are several findings in the US examination that point Tembo as a sub-fertile bull. Although the seminal vesicles were nicely full, which means that the animal was capable of breeding and ejaculate, they presented deposits of protein, which can be due to several reasons: that the animal is having a diet with mineral rich hay, is an old animal, or because ejaculation does not occur often. This evidence, correlated with the empty *ampulla*, is a strong indicator of aspermia in an adult male, which was after proven with the samples obtained by manual stimulation. The bladder presented also signs of protein deposits, probably due to the same reasons (mineral rich hay and/or an old animal).

Reproductive findings in females

Both females presented evidences of urethral stones/calcifications in the beginning (caudal part) of the urethra. Kala was known to have had an episode of cystitis, which could facilitate its formation. It is suggested that if this lesion is present, animals should be closely monitored to diagnose a possible metabolic disease, as males and females were already reported to have urethral obstruction due to calculus, which required surgical intervention (Hildebrandt et al. 2000b; Thongtip et al., 2016).

The vaginal and ovarian findings in Delhi suggested that she was in an early follicular phase when accessed, but although cycling, the female presented ovarian and endometrial cysts which, associated with her previous history of dystocia and birth complications, lead to her being removed from the assisted breeding program. The female is not down regulated at the moment, but will not be a candidate for AI in the future.

There are evidences in other domestic species and other mega vertebrate species (rhinos), that the ovarian down regulation with this vaccine reduces tumour size in the reproductive tract (Hermes et al., 2016). However, few studies have been done in female elephants and is an area of needed research (Lueders & Oerke, 2016). It is important to notice that in “Kala” it was able to control the bleeding caused by the tumour. The vaginal sticky white line found in the mucosa is also an effect of the Improvac® administration, but no deleterious signs of this finding are known.

Chapter 8 Conclusion and Perspectives

Final Considerations

With the inability to sustain the captive elephant populations through imports from the wild, with insufficient captive offspring production and the decline of elephant numbers in their natural habitats, research is urged to gain a better knowledge in how to manage these species' reproduction (Hildebrandt et al., 2012).

Over the last fifteen years, reproductive management of elephants under human care have achieved great improvements. The use of ultrasound, immunoassays and new technologies in wildlife medicine were important and decisive marks in the progression of reproductive physiology understanding in elephants.

Unfortunately, reproduction in elephants in zoos is still not self-sustained, and zoo conservationists and managers are nowadays facing a geriatric and reproductively quiescent, endangered population (Hermes et al., 2004). Zoos face major concerns, as few proven breeding bulls are available, many are not producing good quality of sperm as well as a lack of sexual interest from both elephant genders. Females are aging and developing pathologies that may impede normal cycling and conception (Brown, 2000). Another con is the transport of the animals between zoos for breeding purposes, in order to increase the genetic diversity. The process is an extremely stressful event for the transported individual and also for both the origin and receiving herd due to the highly social character of these species (Brown, 2000). Furthermore, the logistics can get very expensive.

To overcome some of the encountered problems, like the low number of animals per group, related to the limited physical space available for the captive populations, the unpredictability of semen quality, the unpractical collection of *in vivo* oocyte or embryo due to the large size of elephants, mobilization of gametes is an important alternative route. For 15 years, reproduction experts have been using this approach, with fresh chilled semen, even in intercontinental situations, and several pregnancies were achieved. However, the technique depends on frequent hormonal profiling of the estrous cycle and collection has to be performed near ovulation time, otherwise it can lead to a long delay in conception. Due to its availability in place and time and its predictable quality, cryopreserved semen became an important tool and great aid for a more effective AI (Hermes et al., 2013). The above mentioned assisted reproductive technologies, which have been used already for many years in human medicine and domestic animals, will be of great matter to deal with reproductive impediments and maximize reproductive efficiency, consequently allowing advanced breeding programmes to maintain and improve the genetic diversity of the isolated elephant populations, both in the wild and in captivity (Hildebrandt et al., 2006).

A breeding program involving frequent hormonal and ultrasound assessments of male and female elephants, combined with improved management of natural breeding as well as use of assisted reproductive technologies may finally result in the desired creation of self-sustaining elephant captive populations (Hildebrandt et al., 2000b).

Finally, it is more and more crucial to reduce extrapolating data across Asian and African elephants; there are many similarities in endocrine function between these species, but also sufficient differences to propel new comparative studies on all aspects of reproductive biology (Brown, 2006).

Elephants in zoos are often indicated as suffering an in-humane treatment to satisfy humans' selfishness (http://www.peta.org/issues/animals-in-entertainment/zoos/get-elephants-zoos/). With pressure from activism groups, demanding to stop breeding elephant programs, there is a need to deeply understand the role of Zoos nowadays in species conservation. Zoological collections are important to build up public awareness for conservation, to support wildlife conservation research, and are a source of genetic pool for many species. Several species became extinct in the wild, and trough zoo animal collections, breeding of these animals was accomplished and managed to finally reintroduce them in their natural habitats. The Iberian Lynx, the black-footed ferret and the red wolf are some examples of dozens of others with a successful or ongoing European and worldwide reintroduced status (http://www.iberlinx.com;http://blackfootedferret.org/reintroduction; https://www.aza.org/reintroduction-programs). Zoos are a source of species variability, the true "Noah´s Ark" in case of catastrophe. To avoid this extreme scenario all efforts should be made in time to guaranty good breeding programs and management, in captivity and also free ranging elephants, in order to avoid extinction of one more magnificent species.

The main goals established for this work were accomplished, and, with the termination of it, a final conscience of the crucial need for further research and investment towards better understanding the yet unknown aspects of elephant breeding. With the compilation of the most relevant and recent literature in the reproductive management field, the prospect for this dissertation is that it would become an introductory guide and consultation source for the basic steps and methodologies available for elephant reproductive management, and as an orientation for veterinary students and zoo veterinarians that currently handle or may want to take care of elephants.

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APPENDIX I – Nutrient concentration for gestating females and calves.

Table 13: Table presenting the minimum nutrient concentrations (DM basis) proposed for elephant diets (based largely on extrapolation from nutrient requirements of horses) (Adapted from: http://www.zutrition.com/elephant-nutrition-guide; Olson, 2004c).

Nutrient	Breeding and early pregnancy	Late pregnancy	Lactation	Growth of juveniles
Crude protein, %	8-10[a]	12	12-14[b]	12-14[c]
Lysine, %	0.3	0.4	0.4-0.5	0.5-0.6
Calcium, %	0.3	0.5	0.5	0.5-0.7
Phosphorous, %	0.2	0.3	0.3	0.3-0.4
Magnesium, %	0.1	0.1	0.1	0.1
Potassium, %	0.4	0.4	0.5	0.4
Sodium, %	0.1	0.1	0.1	0.1
Sulfur, %	0.15	0.15	0.15	0.15
Iron, ppm	50	50	50	50
Copper, ppm	10	10	10	10
Manganese, ppm	40	40	40	40
Zinc, ppm	40	40	40	40
Cobalt, ppm	0.1	0.1	0.1	0.1
Iodine, ppm	0.6	0.6	0.6	0.6
Selenium, ppm	0.2	0.2	0.2	0.2
Vitamin A, IU/kg	3,000	3,000	3,000	3,000
Vitamin D, IU/kg	800	800	800	800
Vitamin E, IU/kg	100	100	100	100
Thiamin, ppm	3	3	3	3
Riboflavin, ppm	3	3	3	3

a. Adult maintenance, 8% CP; breeding bull, pregnant cow (1st two-thirds of pregnancy), 10% CP.
b. First year of lactation, 14% CP; 2nd year of lactation, 12% CP.
c. Weanling, 14% CP; 3-yr-old, 13% CP; 4-yr-old to 12-yr-old, 12% CP.

APPENDIX II – Lisbon Zoo Anaesthesia Report.

Anaesthesia report from “Jasa” during the sedation for semen collection recorded and performed at Lisbon Zoo (Cortesy of Dr Teresa L. Fernandes, Lisbon Zoo):

Species: Loxodonta africana africana, South African bush elephant
Age: 16 years 3 months 3 days   Birth: 6 of August, 1997.
Date of anesthesia: 8 of November, 2013.
Health: Normal - class I
Environmental temp: 18^{0}C
Preanesthetic Fasting: < 8 hours
Activity: active and alert.
Immobilized in the small enclosure (as an isolated animal).
Body weight: 4000 Kg (8818 Lb) Est.
Body condition: good / normal
Initial dose given at 8h 42m
Showed initial drug effects at 10 min (8h52m)
No anaesthetic complications.
Anaesthetic induction was poor.
Muscle relaxation was poor.
Overall rating for anaesthesia: poor.
Recovery Data: Recovery was normal.
Recovered to normal by 11h30m.

APPENDIX III – Sedation protocols

Table 14: Sedation protocol used in Lisbon Zoo, during the semen collection attempt of the male "Jasa" (Courtesy of Dr Teresa L. Fernandes, Lisbon Zoo).

Drug used		**Time**	**Effect**	**Dose**		**Via**
Butorphanol	67.5mg	8:42	Mild sedation at 10min	16.875mcg/kg	Non-metal Dart	IM
Detomidine	67.5mg	8:42	Mild sedation at10min	16.875mcg/kg	Non-metal Dart	IM
Butorphanol	20mg	31 min	Mild sedation	5.000mcg/kg	Blowdart	IM
Detomidine	20mg	31 min	Mild sedation	5.000mcg/kg	Blowdart	IM
Butorphanol	20mg	47 min	Mild sedation	5.000mcg/kg	Blowdart	IM
Detomidine	25mg	47 min	Mild sedation	6.250mcg/kg	Blowdart	IM
Butorphanol	20mg	76 min	Mild sedation	5.000mcg/kg	Blowdart	IM
Detomidine	20mg	76 min	Mild sedation	5.000mcg/kg	Blowdart	IM
Atipamezole	10mL	138min	Fully recovered at 2min		Non-metal Dart	IM
Naltrexone	7mL	138min	Fully recovered at 2min		Non-metal Dart	IM

min – minutes

Some considerations on the sedation of "Jasa" can be made (personal communication with Lisbon Zoo Veterinarians, 2013): The initial dosage should have been higher or a bigger amount of reinforcement in the beginning. The animal was sedated but never in a sufficient status to allow entrance in the box or even to access IV in the hear vein. The procedure had some probable related ccomplications since three days after the sedation; "Jasa" presented sporadic light to moderate colics episodes. The treatment consisted in exercise, alteration of the diet and administration of pain killer drugs when necessary.

Figure 63: Most commonly used drugs and dosages in African and Asian elephants for sedation and immobilization (Source: Wiedner, 2015).

Doses for Sedating and Immobilizing Elephants

Drug	Dose*	Species†	Route	Notes
Azaperone[21]	0.024–0.038	E.m.	Intramuscularly (IM)	Short-acting tranquilizer
Azaperone[21]	0.056–0.107	L.a.	IM, intravenously (IV)	Short-acting tranquilizer
Carfentanil[21]	0.002–0.004	E.m.	IM	Immobilization agent
Carfentanil[21]	0.0013–0.0024	L.a.	IM	Immobilization agent
Detomidine plus Butorphanol[47]	0.013–0.02 0.013–0.02	L.a.	IM	Standing sedation
Detomidine plus Butorphanol[63]	0.02–0.03 0.02–0.03	E.m. young calf	IM	Standing sedation, but may cause very young elephants to lie down
Etorphine[21]	0.002–0.004	E.m.	IM	Immobilization agent
Etorphine[21]	0.0015–0.003	L.a.	IM	Immobilization agent
Medetomidine[21]	0.003–0.005	E.m.	IM	Sedative
Xylazine[21]	0.04–0.08	E.m.	IM	Sedative
Xylazine[21]	0.08–0.1	L.a.	IM	Sedative
Xylazine plus Butorphanol[21]	Xylazine: 0.035–0.16; Butorphanol[21]: 0.005–0.036	L.a.	IM or IV	Can give separately with xylazine IM first followed by butorphanol, IV, 20 minutes later, or together IV
Xylazine plus Ketamine[21]	Xylazine: 0.1, Ketamine 0.3–0.7	E.m.	IM	

*Doses are in milligram per kilogram (mg/kg) unless otherwise stated.
†E.m., *Elephas maximus*; L.a., *Loxodonta africana*.

APPENDIX IV – *Loxodonta africana* captive herd at Lisbon Zoo.

Table 15: Elephant captive herd of Lisbon Zoo, ZIMS Data on the Taxon Report *Loxodonta africana,* acceded on 9 December 2013 (Courtesy of Dr Rui Bernardino, Lisbon Zoo, Portugal).

Gender	Name	Age*	Origin	Origin	Birth date	Death date	Comments
Male	John	21y	Wild born	Kruger park	18-09-1989	22-20-2010	
Female	Jane	24y	Wild born	Kruger park	18-09-1989		
Female	Nina	23y	Wild born	Kruger park	04-09-1990		
Female	Luna	23y	Wild born	Kruger park	04-09-1990		
Male	Jasa	16y	Captive born	Howletts Wild Animal Park	06-08-1997		Two loans **
Female	-	5d	Captive born	Lisbon Zoo	16-04-2003	21-04-2003	Dam: Nina Sire: John
Male	Trombinhas	3y	Captive born	Lisbon Zoo	01-01-2004	10-01-2007	Dam: Jane Sire: John
Female	Primavera	8y 8m	Captive born	Lisbon Zoo	20-03-2005		Dam: Nina Sire: John
Female	Assunção	7y 6m	Captive born	Lisbon Zoo	25-05-2006		Dam: Luna Sire: John

* y – Years, m – Months, d – Days.
** Loan to Terra Natura in 07-12-2005 and loan to Lisbon Zoo in 08-04-2011.

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